DOES NOT CIRCULATE

VOL. XXXVI. No. 1] [FEBRUARY, 1955

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

UNIVERSITY OF MICHIGAN
MAR 29 1955
MEDICAL LIBRARY

EDITOR
L. P. Garrod

ASSISTANT EDITORS
C. F. Barwell, H. Lehmann, R. A. Shooter

ADVISORY EDITORS
M. P. Curwen, J. R. Marrack, A. A. Miles, W. T. J. Morgan, C. G. Pope
R. W. Scarff, Wilson Smith, D. D. Woods

with the support of

S. P. Bedson, B. C. Dodds, C. L. Evans, H. W. Florey, H. D. Kay,
J. W. McLeod, W. J. Tulloch

DIRECTOR
Paul Fildes

LONDON
H. K. LEWIS & Co. Ltd.
136 GOWER ST., W.C.1

on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C.2

Annual Subscription, January to December, post free, Four Pounds net
Single Numbers, Sixteen Shillings net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor L. P. GARROD, Department of Pathology, St. Bartholomew's Hospital, London, E.C. 1.

General Correspondence relating to the conduct of the Journal should be addressed to Sir PAUL FILDES, Sir William Dunn School of Pathology, University of Oxford.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—Every endeavour is made to publish papers as soon as possible, but under present conditions a delay of 4–6 months between receipt and publication can rarely be avoided.

Proofs.—Proofs will in future be submitted to authors in any part of the world. Prompt return by air mail from distant contributors is necessary if publication is not to be delayed.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers *before the issue is printed.*

Estimates for reprints will be supplied on request to the Publishers.

Made and printed in Great Britain.

Vol. XXXV, No. 6 (December, 1954) was issued on 7.i.55.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXXVI FEBRUARY, 1955 NO. 1

THE DOUBLE DIFFUSION PRECIPITIN TECHNIQUE AS A TOOL FOR THE STUDY OF THE INDUCTION PERIOD OF ANTIBODY FORMATION*

R. K. JENNINGS AND FRANCES MALONE.

From the Rheumatic Fever Research Institute, Northwestern University, Chicago.

Received for publication September 24, 1954.

ANALYTICAL methods providing means of following the disappearance of injected antigen as well as the subsequent rise in antibody content of the blood may be of value in many research applications. In addition, the information they afford may be required in establishing optimal immunisation procedures for the production of high titre sera. While several such methods are already available, recently developed diffusion-precipitin techniques possess characteristics which may offer certain advantages. Thus, the method described below permits simultaneous analysis of a single small sample of blood for both precipitin and free residual precipitinogen. Roughly quantitative estimates may be available in a few hours, in most cases, permitting decisions as to the advisability of further injections or bleedings the same working day. No special " label ", such as radioactivity, other than the specific determinant group responsible for the precipitin reaction itself is needed for the assay of free residual antigen in the circulating serum.

Except for the time element, the familiar Ouchterlony plate method (see review by Oudin, 1952) could be used in the same manner as is described below for the " triangle-plate " modification (Jennings and Malone, 1954) used in our laboratory. It is to be expected that precipitate patterns of analogous form will be encountered by investigators employing the Ouchterlony plate in experiments involving the use of two serum depôts. The experiments described here will, it is hoped, assist in the proper interpretation of such findings.

MATERIALS AND METHODS.

Lucite plates, whose walls form internally a 50 mm. equilateral triangle, are filled with 4 ml. of 1 per cent clarified agar in buffered saline containing merthiolate. A razor-blade, cut to the proper length to fit between notches provided in the middle of each wall, is used to isolate one corner area at a time. The agar in this area is replaced with 0·9 ml. of one of the three reagents—unknown serum, indicator serum or reference antigen—in 1 per cent agar. When this has hardened, the process is repeated in each remaining corner with another of the reagents.

* This investigation was supported by a contract (N7onr-450) between the Office of Naval Research and Northwestern University.

That is to say, melted 2 per cent agar in saline is diluted with an equal volume of reagent to form 1 per cent agar. Each depôt represents roughly one-quarter of the original volume of agar. Thus one plate requires but 0·5 ml. of unknown, and it has been found possible to employ whole or defibrinated blood rather than separated serum when necessary. If further conservation of reagents is desirable, it may be accomplished at the cost of a slightly longer observation period by using half the thickness of agar and correspondingly smaller volumes of reagents.

Since the three depôts in such a plate are essentially contiguous, diffusion immediately leads to an overlapping of gradients at the corners of the central triangular reaction arena. If these gradients contain concentrations of antigen and antibody greater than a threshold value, zones will be formed. The time required is dependent largely on the factors which determine the *Kf* value of the particular system.

Using, as the unknown, serum from an animal which has received an injection of antigen, free antigen may remain in the specimen at the time of bleeding or antibody production may have progressed sufficiently so that free precipitin is present. If uncombined antigen remains, it will be available for reaction with antibody supplied by the indicator serum depôt. If antibody is in excess, the known antigen employed in the third depôt becomes the indicator reagent and a zone will appear at the opposite corner of the reaction arena from that which would have been the case if antigen had been present.

Thus the location of the zone provides a quite unequivocal demonstration of the condition of the unknown in respect to free antigen or antibody within a few hours. If the identity of a precipitin system is doubtful, zone growth may be permitted to continue until the reference zone, which will form in any case between the two known reagent depôts, is met. Coalescence or crossing will demonstrate the specificity or otherwise of the zone in question.

Reagents.

Observations were made on serum specimens withdrawn at intervals from rabbits undergoing hyperimmunisation for the production of antisera to be used in other experiments. Bovines erum albumin (BSA) was employed as a 1 per cent solution of Fraction V (Armour) in buffered saline. Control tests with crystalline bovine albumin and with antiserum prepared against the crystalline product indicated that the precipitin system thus studied involved an antigen present in the crystalline preparation rather than any possible adulterant peculiar to the less pure Fraction V.

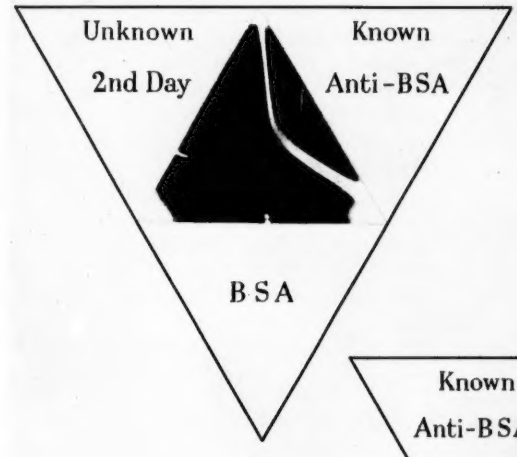
A second series of rabbits was immunised with whole guinea-pig serum as antigen, in the course of experiments undertaken by C. E. French of this Institute. He generously supplied us with individual samples of the sera of several of these animals and with pooled sera from two others. These samples were taken 5 days after the last injection of a series of daily intravenous injections. Following preliminary experiments, two were chosen as indicator reagents, since both of these contained all the antibodies present in any of the sera examined. Fresh whole guinea-pig serum was used as the known antigen solution.

EXPLANATION OF PLATE.

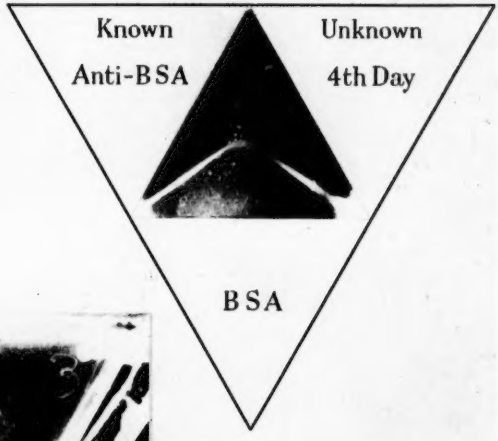
FIG. 1.—Antigen excess and antibody excess in serum.

1*a*. Serum taken 2 days after administering antigen.	1*b*. Serum taken 4 days after administering antigen.
Upper left, unknown serum (F_2).	Upper right, unknown serum (F_3).
Upper right, known anti-BSA (B).	Upper left, known anti-BSA.
Bottom, 0·01 per cent BSA (Fraction V).	Bottom, 0·01 per cent BSA (Fraction V).

FIG. 3.—Pattern formed by antisera from two rabbits injected with whole guinea-pig serum.
Upper left, serum, rabbit 4.
Upper right, serum, rabbit 3.
Bottom, whole guinea-pig serum.
Photographed at end of 24 hr. at room temperature.
Zones B and C too faint to record photographically.

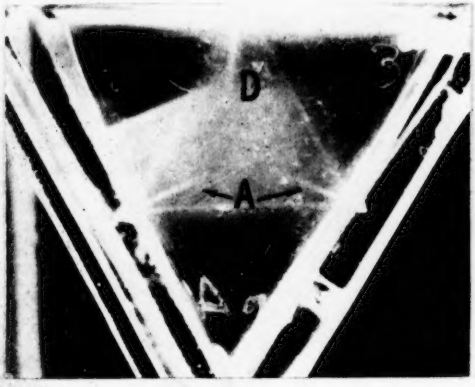

Unknown
2nd Day
Known
Anti-BSA
BSA

1a


Known
Anti-BSA
Unknown
4th Day
BSA

1b

3


D
A

RESULTS.

Patterns of antigen and of antibody excess.

Two rabbits used for anti-BSA production some months previously have been chosen as typifying different individual responses to a particular stimulus. Preliminary examination failed to produce evidence of the presence of detectable amounts of precipitin or antigen in either serum. Each rabbit was then given a single intraperitoneal injection of 5 ml. of a 1 per cent solution of BSA in buffered saline. Two days later a blood sample was withdrawn from an ear vein and a second injection of 1 ml. of 1 per cent antigen was administered intravenously. Small blood samples were withdrawn without further antigen injection 2, 4, 6, 9 and 11 days later.

Free antigen was found in the blood of both animals the second day after the intraperitoneal injection and on the second day after the intravenous injection. Two days later (the fourth day after antigen administration) free antibody was found in the serum of rabbit F, as shown by the patterns reproduced in Fig. 1. Rabbit E, on the other hand, continued to show an excess of free antigen 8 days after the last injection. Thereafter neither antigen nor antibody could be demonstrated in the serum of this animal. A further series of injections of Fraction V a month later finally elicited some antibody production, but the antibody was directed against another antigenic component of the BSA preparation as shown by failure of the zones to coalesce with zones formed by serum from rabbit F.

Estimation of the amount of antigen in serum.

The present state of development of the triangle plate method will permit only a rough estimate of antigen concentration to be made on a comparative basis. The breadth of the reference zone formed when, for example, 0·1 per cent BSA was used as reference antigen is suggested in Fig. 2 in comparison with the pattern obtained when the reference antigen concentration was one-tenth as great. It is apparent that the breadth of a zone at its base is dependent on the reagent

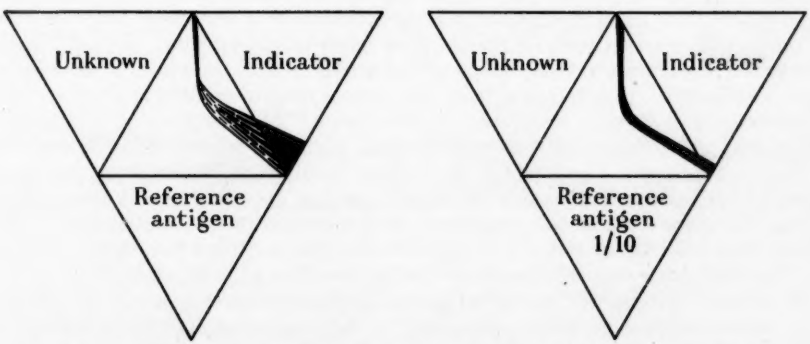


FIG. 2.—Adjustment of concentration of reference reagents to limit zone migration.

Upper left, unknown (Rabbit F, 2 days).
Upper right, reference anti-BSA.
Bottom, 0·1 per cent BSA.

Upper left, unknown (Rabbit E, 2 days).
Upper right, reference anti-BSA.
Bottom, 0·01 per cent BSA.

concentration, all else being equal. Other features of the pattern may reflect the same relationship, possibly more exactly (Jennings and Malone, 1954), but suitable means of measuring them are still under consideration.

Consistent with the fact that the unknown in the experiment from which Fig. 2A was constructed represented the last specimen from rabbit F before the appearance of antibody, this zone was narrower than the corresponding one represented in Fig. 2B. The unknown in the latter preparation was taken from rabbit E at the same time, and it has been pointed out that elimination of antigen was much slower in this animal.

When possible, the known antigen and antibody solutions may be balanced by trial and error so that a narrow non-migrating zone is formed. Such a zone is maximally dense and has the further advantage that any slight widening of the unknown zone either into the unknown or into the indicator depôt becomes readily apparent by contrast. This provides a reliable means of determining whether the antigen remaining in the unknown is greater or less than the concentration used in the reference standard.

Similar evidence in regard to the antibody titre will be observed, but must be employed with greater caution. It has been pointed out (Jennings and Malone, 1954) that the rate of antibody diffusion is probably influenced by the total globulin concentration in the serum rather than the antibody concentration alone, and there is no assurance that this will remain constant from one bleeding to the next, nor that it will equal the globulin concentration in the reference serum.

Patterns formed by multiple precipitin systems.

A maximum of four zones was observed with the guinea-pig antigens and corresponding rabbit antibodies. Disregarding the possibility that zones may have been superimposed in these patterns or that a single antigen or antibody might have participated in more than one precipitin system, the results will be described as though it were known that each zone represented a single antigen and a single antibody distinct from each of the others.

The practice of balancing the reference system cannot be followed when several reagents are involved. Since migration of some of the zones was quite pronounced, the total precipitate was dispersed over a relatively large area, and photographic records of the patterns were quite unsatisfactory. Fig. 3 has been chosen to illustrate the complexity of the patterns and the effect of zone migration. Otherwise, results have been illustrated diagrammatically in Fig. 4. In the interests of clarity, only the "leading edge" of each zone is shown in the diagrams. The diagrams were drawn from plates in which diffusion had been allowed to proceed long enough to permit coalescence, in order to verify the specificities involved. In practice it is better to use shorter diffusion periods, especially when considerable migration is involved, in order to minimise artefacts which may arise as a result of temperature changes and other uncontrolled factors.

The four antigen-antibody reactions indicated in Fig. 4A were observed with both serum 4 and serum 5, tested against normal guinea-pig serum as indicator. The reactants forming these zones will be referred to alphabetically, beginning with the zones which appeared nearest to the indicator depôt.

Of the four antibodies present in serum 4 and 5, only A and B were found in serum 3, while serum 2/7, a pool from two rabbits, contained only A. Consequently, when serum 3 and serum 2/7 were tested simultaneously with guinea-pig

serum as the source of indicator reagents, only the three zones shown in Fig. 4B were observed.

However, when serum 3 or serum 2/7 was tested against either serum 4 or 5 as a reference reagent, zones were observed to grow from all three corners of the reaction arena, as shown in Fig. 3, 4C and 4D. Coalescence of the zone with zone D of the reference system showed that this antigen was still in excess in each of these two unknowns. In spite of the absence of antibody C from either serum 3

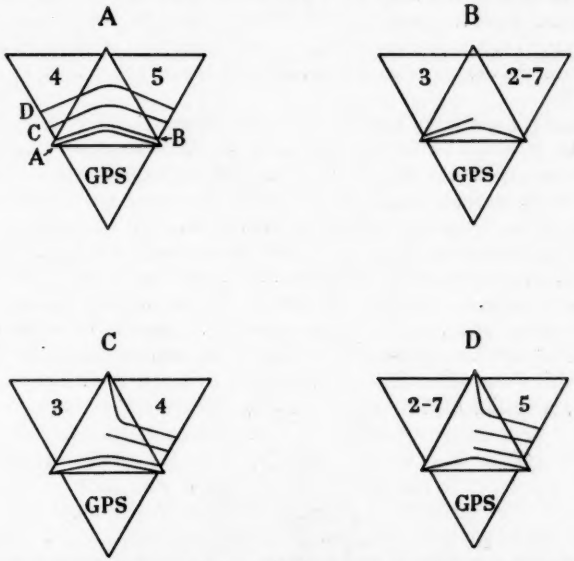


FIG. 4.—Patterns produced by various individual antisera from group of rabbits immunised with guinea-pig serum.

A.
Upper left, serum 4.
Upper right, serum 5.
Bottom, guinea-pig serum.

B.
Upper left, serum 3.
Upper right, serum 2–7.
Bottom, guinea-pig serum.

C.
Upper left, serum 3.
Upper right, serum 4.
Bottom, guinea-pig serum.

D.
Upper left, serum 2–7.
Upper right, serum 5.
Bottom, guinea-pig serum.

Drawings made at end of 48 hr. at room temperature.

Zones illustrated as leading edges, only, disregarding precipitate trail along path of migration (see Fig. 3). (Note. Serum 3 *vs.* serum 5, and serum 2–7 *vs.* serum 4 gave patterns indistinguishable from those illustrated as C and D.)

or serum 2/7, no evidence of an excess of antigens for these two systems was observed. Sera taken from the animals at a later bleeding were found to contain all four antibodies in each case.

DISCUSSION.

Four components of the guinea-pig serum were capable of eliciting an antibody response in all the rabbits tested. Due, apparently, to differences in individual

response to the same stimulus, the time at which the various antibodies appeared in the sera varied from animal to animal. Rabbit E of the BSA series appeared to be incapable of responding at all to a stimulus which led to prompt antibody production in rabbit F. Such individual variations are known to be frequent. The experiments described suggest a screening method for the elimination of non-reactors or poor reactors from a group of animals chosen for antibody production.

Dixon, Maurer and Deichmiller (1954) have pointed out that the first evidence of immune response is a rapid drop in residual antigen. The distinction between an animal giving such a response and a " non-reactor " was even more apparent, in this respect, on direct observation of the zones than it is in comparison of Fig. 2A and 2B.

The dramatic switch in location of zones from one bleeding to the next, in animals which produce sufficient antibody to overwhelm the antigen, serves to emphasise facts which are known but may be neglected when evidence of their existence is rarely encountered. It is readily apparent that antibody production is rapid at the time when free antibody first appears in the serum. The absolute titre of antibody at a given moment, or the time required to achieve a chosen level, is seen to be an unsatisfactory criterion of " good" or " bad " antigens. We do not know, for instance, whether antigen A, for which antibody appeared in all the sera examined, was really a more powerful stimulus of antibody production than antigen D of the guinea-pig proteins, or whether antigen D was simply considerably more concentrated and presented a higher blood level to be overcome. Heidelberger, Kendall and Soo Hoo (1933) reported that large doses of antigen led to delayed appearance of antibody. In this connection it should be pointed out that either serum 2 or serum 7 may have contained free antibody C, masked by free antigen present in the serum with which it was mixed. Such a possibility should be taken into account in judging antisera on the basis of pooled aliquots.

It is to be regretted that the method employed has not yet been developed to the point of exact quantitative expression. This limitation makes it undesirable at present to discuss the period of antigen-antibody balance. The presence of a rather high concentration of antigen-antibody complex in the serum at this stage and for some time thereafter (Sternberger, Maltaner and Deweerdt, 1953) is a matter which must soon receive consideration in relation to its possible effect on the interpretation of diffusion-precipitin test results.

SUMMARY.

If a sample of serum withdrawn from a rabbit after parenteral administration of an antigen be placed in one depôt of a double-diffusion plate, a known solution of the antigen in a second depôt and a known antibody solution in a third, it becomes possible to test for an excess of antigen or of antibody simultaneously with the single sample of unknown. If antigen is still present, the zone will form between the depôt containing the serum to be analysed and that containing the known antibody. If antibody is in excess, the zone will form between the unknown serum and the known antigen depôts. When the triangle plate modification of the Ouchterlony method is used the distinction may become apparent in as little as one hour. It is also possible to adjust the concentrations in the

reference system to permit a rough quantitative estimate of the amount of antigen remaining in the unknown.

With whole serum used as the antigen source, patterns were observed in which antibody excess for certain antigens could be recognised while an excess of antigen for other precipitin systems was still present. Individual variation in response to the same course of immunisation was also made apparent by this technique.

REFERENCES.

DIXON, F. J., MAURER, P. H. AND DEICHMILLER, M. P.—(1954) *J. Immunol.*, **72**, 179.

HEIDELBERGER, M., KENDALL, F. R. AND SOO HOO, C. M.—(1933) *J. exp. Med.*, **58**, 137.

JENNINGS, R. K. AND MALONE, F.—(1954) *J. Immunol.*, **72**, 411.

OUDIN, J.—(1952) In 'Methods in Medical Research.' Vol. 5. Chicago (The Year Book Publishers, Inc.), p. 360.

STERNBERGER, L. A., MALTANER, F. AND DEWEERDT, J.—(1953) *J. exp. Med.*, **98**, 451.

STUDIES ON THE VIRULENCE OF TUBERCLE BACILLI. THE EFFECT OF CORD FACTOR ON MURINE TUBERCULOSIS.

H. BLOCH AND H. NOLL.

From the Division of Tuberculosis, the Public Health Research Institute of the City of New York, Inc., New York 9, N.Y.

Received for publication September 27, 1954.

THE term " cord factor " has been previously suggested to designate a bacterial constituent extracted from cultures of tubercle bacilli characterised by the formation of " cords " under proper conditions *in vitro* (Bloch, 1950*a*). This growth pattern is typical of virulent and certain attenuated strains of *Mycobacterium tuberculosis* (Middlebrook, Dubos and Pierce, 1947). The chemistry of cord factor has been extensively studied (Bloch, Sorkin and Erlenmeyer, 1953 ; Noll and Bloch, 1953 ; Asselineau, Bloch and Lederer, 1953). It is an ester of mycolic acid, glucose and a small nitrogen-containing fragment (Noll, 1953 ; Noll and Bloch, 1955). Uniform preparations of a high degree of purity have been obtained from a number of virulent human and bovine strains of tubercle bacilli. The preparations used in the present study were extracted from various batches of the human strains H37Rv and " Brévanne." Their chemical identity and purity have been ascertained by combustion analysis, infra-red spectrum and optical rotation. These values are highly characteristic (Noll and Bloch, 1955).

The present study attempts to find direct evidence for the rôle played by cord factor in the course of a tuberculous infection. It was originally suggested that this bacterial component was concerned in the virulence of the tubercle bacillus (Bloch, 1950*a*). Subsequently, a number of observations and experimental findings have been published in support of this hypothesis (Bloch, 1950*b* ; Bloch and Noll, 1953*a*, *b*). The evidence was circumstantial rather than direct. It was based on suggestive correlations between the amounts of cord factor produced by tubercle bacilli and their virulence under various experimental conditions. This paper describes the direct influence of cord factor injections on experimental tuberculosis, with particular emphasis on the specificity of the observed effects.

MATERIAL AND METHODS.

Cord factor.—The material used had the following chemical specifications. Melting point : 38–42°. Acidity : 1·0 mm.^{3} 0·100 N–CH_3ONa/mg. Optical rotation : $[\alpha]_D^{25}$ 29–30° (c = 0·9, in benzene). Elementary analysis : C 76·5 per cent, H 12·5 per cent, N 0·9 per cent. Infra red spectrum : absorption maxima at : 3350, 2900, 1715, 1475, 1380, 1275, 1170, 1150, 1100, 1075, 1050, 1020, 995, 940, 915, 805, 720 cm.$^{-1}$.

Animals.—Two strains of mice were used : CF1 albinos, from Carworth Farms, New City, N.Y., and C57 Blacks, from Jackson Memorial Laboratories, Bar Harbor, Me. All were males, weighing 15–20 g. They were fed a standard diet of pellets (Rockland Mouse Diet) and water, and caged in groups of not more than 10 per cage. The rabbits were light-skinned males of a mixed breed, from Carworth Farms.

Mode of infection.—The mice were infected intravenously with a standard strain of tubercle bacilli (H37Rv) obtained from The Trudeau Laboratory, Saranac Lake, N.Y. The bacteria

were propagated and injected in liquid Tween-albumin medium (Dubos and Middlebrook, 1947), unless stated otherwise. Strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* Type III, and haemolytic streptococci were received through the courtesy of the Department of Bacteriology at New York University. They were kept in the dry-frozen state and propagated before use in beef-heart broth containing about 5 per cent horse blood.

EXPERIMENTAL.

Toxicity of Cord Factor for Mice.

It has been previously stated that in order to show its characteristic toxicity for mice, cord factor has to be injected repeatedly and at intervals (Bloch, 1950*a*). This fact has since been confirmed in numerous tests and the technique described in detail (Bloch *et al.*, 1953; Bloch and Noll, 1953*a*). With the preparations used in the present experiments, 5 injections of 5 μg. each, suspended in 0·05 to 0·2 ml. light mineral oil (Bayol F) or olive oil and spaced 2 to 3 days apart from one another, killed at least 50 per cent of the mice between 7 and 28 days following the first injection, whereas a single injection of up to 1 mg. failed to kill. Typical data of such a test have been published (Bloch *et al.*, 1953). Each injection was followed by a severe drop in weight and, as a rule, the spacing of the repeated injections was guided by the weight changes of the mice, subsequent injections being given when the animals had regained their weight before the previous injection. The significance of the time interval was not known.

TABLE I.—*Effect of Five Injections of* 0·01 *mg. Cord Factor, given at Different Time Intervals.*

Intervals between injections.	Individual survival times (days after the first injection) (Ten C57 Black mice per group).
1 day . . .	4, 8, 8, 8, 10, 11, 11, 11, 19, 21
2 days . . .	8, 8, 9, 9, 9, 11, 14, 16, 17, 18
4 ,, . . .	12, 13, 15, 16, 16, 20, 22, 39, 42, S*
8 ,, . . .	17, 25, 35, 38, 43, S, S, S, S, S
14 ,, . . .	S, S, S, S, S, S, S, S, S, S

* S indicates mouse was sacrificed 80 days after the first injection, when the experiment was discontinued.

In the following experiment (Table I), groups of C57 Black mice received equal doses of cord factor, but the injections were given at different time intervals. Every group was injected 5 times with 0·01 mg. cord factor. Table I shows that there was no significant difference between the groups injected daily, every other day and every 4th day. When the injections were spaced 8 days apart, the number of mice dying from these injections decreased markedly, and even more so when 2 weeks were allowed to elapse between injections.

When a preparation of cord factor was tested in progressively decreasing amounts, a clear end point was reached beyond which toxicity was no longer detectable, with either one or repeated injections. Chemically pure preparations were toxic at a minimal dose of 2·5–5 μg. per mouse, injected 5 times successively. However, with doses larger than the minimum, repeated injections were not correspondingly more toxic; *e.g.*, the results of 5 injections of 0·005 mg. of a pure preparation did not differ significantly from the effects of 5 injections of 0·1 mg. of the same material.

The peculiar circumstances in which cord factor was toxic and the gross pathological lesions it produced, were reminiscent to some extent of the Shwartzman phenomenon. Therefore, an experimental attempt was made to determine whether cord factor gave a local or generalised Shwartzman phenomenon. All results were negative. In the rabbit no local or generalised Shwartzman phenomenon could be elicited by two cord factor injections. Likewise, cord factor was ineffective in any combination with meningococcus toxin or with starch. In the mouse, it was not possible to influence the toxicity of cord factor by various doses of nitrogen mustard or thorotrast, substances which affect the outcome of the generalised Shwartzman phenomenon in the rabbit (Stetson and Good, 1951; Good and Thomas, 1952).

Equally unsuccessful were attempts to modify the toxicity of cord factor in the mouse by the following antibiotics and drugs: streptomycin, para-aminosalicylic acid, isoniazid, thiosemicarbazone (TBI), penicillin, Triton A 20 and four other related surface-active agents found to have a therapeutic effect in murine tuberculosis by Cornforth, Hart, Rees and Stock (1951). Rutin (" Vitamin P "), which is thought to reduce capillary permeability, and pyribenzamine likewise had no effect.

Hence, neither by direct nor indirect methods has it been possible to relate the toxicity of cord factor to the type of tissue reaction known as the Shwartzman phenomenon. The ineffectiveness of pyribenzamine seems to indicate that the delayed toxicity is not related to allergic types of reactions, although more extensive experimentation is needed to preclude this possibility. Antituberculous drugs were likewise ineffective in modifying the toxicity of repeated injections of cord factor.

Effect of Cord Factor Injections on Experimental Tuberculosis.

If cord factor is concerned with the virulence of the tubercle bacillus, a combination of cord factor injections and bacterial infection should result in enhancement of the effects of infection. This has been shown to be the case in three types of experiment of which representative examples are illustrated in Tables II and III.

Table II shows an experiment where mice were injected intraperitoneally with one dose of cord factor in Bayol F followed 24 hr. later by an intravenous injection of various dilutions of a 5-day Tween-albumin culture of tubercle bacilli. There were 10 mice per group and 2 sets of infection controls, one receiving an intraperitoneal injection of Bayol F alone 24 hr. before infection, the other no injection prior to infection. The experiment was discontinued and the surviving mice sacrificed 43 days after infection. A similar experiment is shown in Table III, where an injection of cord factor (this time dissolved in olive oil) was combined with infection with various dilutions of a 3-week culture of H37Rv resulting in a chronic type of tuberculosis. It has been previously found (Bloch, 1950*b*) and confirmed (Fenner and Leach, 1953) that bacteria from 3-week old cultures are less virulent than equal numbers of organisms from young cultures of the same strain. In both sets of experiments a single injection of cord factor given one day prior to infection clearly enhanced both types of infection.

Another way of showing the enhancing effect exerted by cord factor on tuberculosis was to follow a technique used by Hart and Rees (1950) in demonstrating

TABLE II.—*Combined Effects in CF1 Mice of Cord Factor Injections and Infection with Various Doses of 5-day-old Cultures of Tubercle Bacilli.*

Group no. (10 mice per group).	Intraperitoneal injection one day before infection.	Infective dose.	Survival times of mice (days).	Median survival time (days).
1	0·1 mg. cord factor*	Undil.†	9, 9, 9, 10, 11, 13, 14, 19, 20, 20	11
2	ditto	1 : 2	9, 9, 9, 12, 15, 15, 16, 18, 19, 19	15
3	„	1 : 4	9, 13, 14, 15, 18, 19, 21, 23, 29, 33	18
4	0·1 ml. Bayol F	Undil.	14, 14, 14, 17, 18, 19, 19, 19, 19, S‡	18
5	ditto	1 : 2	18, 18, 19, 19, 20, 20, 21, 21, 25, S	20
6	„	1 : 4	20, 20, 20, 20, 20, 22, 22, 26, S, S	20
7	—	Undil.	16, 18, 19, 19, 19, 20, 20, 20, 20, S	19
8	—	1 : 2	18, 19, 19, 20, 20, 20, 23, S, S, S	20
9	—	1 : 4	12, 19, 21, 21, 21, 21, 21, 21, 22, S	21
10	0·1 mg. cord factor	None	2, S, S, S, S, S, S, S, S, S	> 43

* 0·1 mg. of cord factor was injected intraperitoneally, dissolved in 0·1 ml. Bayol F.
† The mice were infected intravenously with 0·1 ml. of a 5-day Tween-albumin culture of H37Rv and dilutions thereof, as indicated.
‡ S indicates mouse was sacrificed 43 days after infection, when the experiment was discontinued.

TABLE III.—*Combined Effects in CF1 Mice of Cord Factor Injections and Infection with Various Doses of 3-week-old Cultures of Tubercle Bacilli.*

Group no. (10 mice per group).	Intraperitoneal injection one day before infection.	Infective dose.	Survival times of mice (days).	Median survival time (days).
1	0·1 mg. cord factor*	Undil.†	10, 11, 15, 16, 17, 19, 20, 21, 21, 22	17
2	ditto	1 : 2	12, 13, 14, 18, 21, 22, 23, 26, 28, 29	21
3	„	1 : 4	3, 22, 24, 27, 43, 61, S‡, S, S, S,	43
4	„	1 : 8	7, 18, 19, 31, 42, S, S, S, S, S	42
5	„	1 : 16	S, S, S, S, S, S, S, S, S, S	> 61
6	—	Undil.	19, 21, 23, 31, 38, 45, S, S, S, S	38
7	—	1 : 2	21, 24, 24, 29, 37, S, S, S, S, S	37
8	—	1 : 4	S, S, S, S, S, S, S, S, S, S	> 61
9	—	1 : 8	46, 52, 59, S, S, S, S, S, S, S	> 61
10	—	1 : 16	52, S, S, S, S, S, S, S, S, S	> 61
11	0·1 mg. cord factor	—	S, S, S, S, S, S, S, S, S, S	> 61

* 0·1 mg. cord factor was injected intraperitoneally, dissolved in 0·1 ml. olive oil.
† The mice were infected intravenously with 0·1 ml. of a 1/4 dilution of a 3-week-old Tween-albumin culture of H37Rv and further dilutions thereof as stated.
‡ S indicates mouse was sacrificed 61 days after infection, when the experiment was discontinued.

the enhancing effect of cortisone on chronic tuberculosis in mice. Fifty CF1 mice were injected intravenously with 0·02 ml. of a 3-week culture of H37Rv, producing chronic tuberculosis. The mice were kept together and weighed about 5 weeks after infection. The 30 animals showing the highest weights (ranging from 25 to 33 g.) were caged in equal groups of 10 and treated as follows : one group received 10 subcutaneous injections of 0·1 mg. cortisone over a period of 2 weeks ; a second group received 2 injections of 0·05 mg. cord factor with a 1-week interval between the 2 injections ; the third group was left as infection control. Two more groups of 10 non-infected mice each were added as cord factor and cortisone controls. The experiment was discontinued 120 days after infection, or 86 days after the first cord factor and cortisone injections.

The median survival time for the mice receiving cord factor was 76 days, for the cortisone-treated animals 56 days, and for the untreated controls 109 days. Thus the mice injected with cord factor died sooner than the controls, as did those injected with cortisone. The tuberculous lesions found in the lungs of the two injected groups of mice were very similar, consisting of large round necrotic areas, confluent in some parts, well isolated in others. Histologically, the necrotic areas showed amorphous débris teeming with acid-fast bacteria. While it is not suggested that the same mechanism was operative in the two groups, it appears likely that in both instances death occurred from an activation of existing tuberculous foci with a flare-up of bacterial multiplication and subsequent tissue destruction in these areas. In both groups there was a remarkably weak cellular reaction around the inflammatory centres. It did not seem unlikely that the exacerbation of chronic tuberculosis by the action of cord factor was due to a more rapid bacterial multiplication than occurred in the controls. Hence an experiment was carried out where the bacterial content of lungs, liver and spleen was determined at various times after infection in mice receiving 0·05 mg. cord factor in Bayol F prior to the infection. These values were compared to organ counts from control mice injected with 0·05 ml. of Bayol F alone. All mice were infected intravenously with a large dose of tubercle bacilli (approximately 10^8 infectious units). Three animals of each experimental group were killed by chloroform 1 hr., 2 days, 6 days and 13 days after infection. Lungs, spleens and livers were removed aseptically and the whole organs ground individually with 50 ml. of a 0·5 per cent aqueous albumin solution in a Virtis homogeniser for 30 sec. From these homogenates, subsequent dilutions and plate counts were made. The average number of bacteria cultured from the 3 corresponding organs of the animals of each group was consistently higher in the organs of the cord factor-treated mice. At 48 hr. after infection the differences were about fourfold. The initial drop in bacteria recovered within the first two days was less pronounced in the animals which had received cord factor prior to infection than it was in the control mice. Hence, the subsequent bacterial multiplication started at a higher level and rose faster to a maximum than in the controls. The differences were not of the same order in every organ. The high number of bacteria present in the liver of all mice was remarkable.

The infection-enhancing action of cord factor was effective when the compound was injected as long as 3 days before infection, but given 6 days before infection it had no noticeable effect. Just as there is a threshold dose for cord factor toxicity, the infection-enhancing effect of a single injection is dependent on the dose. Table IV lists an experiment where groups of 10 CF1 mice were given

TABLE IV.—*Effect of Various Doses of Cord Factor given 24 Hours Prior to Tuberculous Infection.**

Group no. (10 mice per group).	Amount of cord factor injected 24 hr. before infection.	Average survival time and range (days).
1	0·1 mg.	12·7 (7–18)
2	0·01 „	16·8 (9–24)
3	0·001 „	17·9 (16–20)
4	—	18·2 (17–20)

* The mice were infected intravenously with 0·1 ml. of a 5-day-old culture of H37Rv.

one injection of 0·1 mg., 0·01 mg. and 0·001 mg. respectively, 24 hr. before infection with a large dose of H37Rv. It is seen that the two lower doses had very little effect, whereas the highest dose clearly shortened the survival time. Control groups of mice receiving the cord factor injection without subsequent infection all survived throughout the observation period. They are not listed in the Table, since the failure of mice to die after one injection has been demonstrated many times before.

Specificity of the infection-enhancing action of cord factor.

The following experiments were done in order to investigate the specificity of the infection-enhancing action, both with respect to the infective agent as well as to cord factor itself. The effect of cord factor on infections with *Staph. aureus*, *Str. pneumoniae* Type III, and *Streptococcus haemolyticus* in mice were studied under exactly the same conditions as had been chosen for the analogous experiments with tubercle bacilli. Strains of relatively low virulence were used in order to have the best possible chance of observing an enhancing effect. Table V shows that cord factor had no effect on any of these infections.

TABLE V.—*Failure of Cord Factor Injections to Enhance the Effects in Mice of Pneumococcal, Staphylococcal and Streptococcal Infections.*

Number of mice per group.	Dilution of 20 hr.-culture with which mice were infected.		No. of mice dying within 5 days after infection.	
			Group injected with 0·1 mg. cord factor prior to infection.	Control group.
15	Pneumococcus	1/10	15	14
15	,,	1/100	7	7
15	,,	1/1000	1	0
15	,,	1/10,000	1	0
10	Staphylococcus	1/10	4	8
10	,,	1/100	2	0
10	,,	1/1000	0	1
10	,,	1/10,000	0	0
10	Streptococcus	1/10	10	9
10	,,	1/100	7	8
10	,,	1/1000	1	0
10	,,	1/10,000	0	0

The specificity of cord factor was also investigated by injecting control groups, prior to tuberculous infection, with 0·1 mg. each of mycolic acid, with lipopolysaccharide (wax D), with a methylester of mycolic acid, and with a non-toxic bacterial fraction very similar to cord factor (Noll and Bloch, 1955). All compounds were derived from the same strain as the cord factor. None of these compounds, which are all chemically related to cord factor, had an enhancing effect on tuberculosis. Hence, within the limits of the experimental evidence obtained, the synergism between cord factor and tuberculosis is specific.

Interaction between cord factor and isoniazid.

It has been mentioned that isoniazid has no effect on the toxicity of cord factor, but it remained to be determined whether the drug would affect a tuber-

culous infection enhanced by cord factor. Three groups of 20 mice each were injected with 0·1 mg. of cord factor in 0·05 ml. Bayol F. On the following day, 2 groups were infected intravenously with 0·1 ml. of a 5-day culture of H37Rv ($1{\cdot}7 \times 10^7$ infectious units per dose). The third was not infected and served as control for cord factor.* Of the two infected groups, one was given a powdered pellet diet containing 0·01 per cent isoniazid for two weeks; the medication was then discontinued. The other served as control for infection combined with cord factor injection. Two additional control groups, each of 20 mice, consisted of infection controls without cord factor or isoniazid, and infection controls given isoniazid. The results are shown in Table VI. The infection-enhancing effect of cord factor was again confirmed in this experiment. More important, it is seen that this enhanced infection was little affected by isoniazid therapy, whereas the control group with no cord factor responded to the drug in the usual fashion. Thus, cord factor appears to interfere with the chemotherapeutic activity of isoniazid. This antagonistic effect between cord factor and isoniazid is dependent on the dosage of both agents. In an experiment similar to the one described in Table VI a 10 times higher dose of isoniazid (corresponding to a daily drug intake of approximately 4 mg./day/mouse) was sufficient to overcome the infection enhanced by cord factor. The antagonism between cord factor and isoniazid was observed *in vivo* only. Attempts failed to show an inhibiting effect of cord factor on the bacteriostatic action of isoniazid in cultures of tubercle bacilli.

TABLE VI.—*Interference of Cord Factor with the Therapeutic Action of Isoniazid.*

Number of mice per group.	Amount of cord factor injected 24 hr. before infection.	Dose of INH* (per cent in food).	Individual survival times (days). (Median survival time in bold type.)
20	0·1 mg.	—	7, 7, 7, 8, 8, 8, 8, 8, 8, **8**, 8, 8, 8, 9, 13, 13, **13**, 13, 17, 19
20	0·1 mg.	0·01	7, 7, 7, 8, 8, 9, 11, 12, **13**, **15**, 15, 15, 16, 19, 21, 31, 32, 37, 40, S†
20	—	—	17, 18, 18, 18, 18, 19, 19, 20, 20, **20**, 20, 20, 20, 21, 21, 21, 21, 23, 28, 32
20	—	0·01	19, 21, 32, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S
			(Median survival time > **50**)

* INH was given as a 0·01 per cent addition to a powdered pellet diet beginning on the day of infection. The medication was discontinued after 2 weeks.

† S indicates mouse was sacrificed 50 days after infection, when the experiment was discontinued.

DISCUSSION.

Previously reported results had suggested that cord factor was concerned in the virulence of the tubercle bacillus. There was close parallelism between production of cord factor by tubercle bacilli and the pathogenicity of the organisms. Such correlation existed not only between different bacterial strains of varying virulence, but likewise between organisms of the same strain grown under varied cultural conditions affecting their virulence. These experimental findings have been recently reviewed (Bloch, 1953). The correlation which

* Not shown in Table VI. No mice of this group died during the observation period.

was consistently found under varied experimental conditions led to the conclusion that the good agreement was more than coincidental and that a definite rôle is played by cord factor in the course of a tuberculous infection. The present experiments were planned to furnish direct evidence bearing on these conclusions.

Before these experiments could be undertaken, it was necessary to have chemically pure products so that the effects observed could be safely attributed to cord factor itself and not to accompanying impurities. This goal has been achieved. Products are available, of which the chemical composition is known except for a small fragment which has not yet been fully identified (Noll and Bloch, 1955). Structurally similar products have been synthesised and were found to have qualitatively similar action (Asselineau, Bloch and Lederer, 1954). Identical cord factor preparations have been obtained in the same reproducible degree of purity from at least five different virulent strains of mammalian tubercle bacilli (Noll and Bloch, 1955). Also, chemically pure and well characterised products appeared necessary in view of the fact that biological activities of one kind or another have been attributed to so many ill-defined bacterial lipids that comparison of the results of different authors has become almost impossible.

The fact that cord factor is toxic has been a great help in assaying extracts of unknown composition, since no other related bacterial constituent with similar toxic properties was found. Although interesting in itself, the phenomenon may have limited biological significance. If cord factor enters the circulation of an animal under natural circumstances, it is always in connection with tuberculous infection and in the presence of tubercle bacilli. Therefore, the experiments where a single injection of cord factor (which as such has no lasting damaging effect) was combined with tuberculous infection have more bearing in a discussion on the rôle of cord factor. In all instances it was clear that experimental tuberculosis takes a more severe course if combined with an injection of cord factor. The injection can precede or follow an acute infection, or it can intervene later during the stationary phase of a mild infection and exacerbate the chronic disease. The degree to which the combination enhances the infection depends on the amount of cord factor injected as well as on the infective dose. As was to be expected, there is a minimal threshold dose of cord factor below which no noticeable enhancement of tuberculosis takes place, while a given effective dose of cord factor will render the mild as well as the severe experimental infections correspondingly more acute. The experimental device of combining injections of cord factor with infection is, of course, artificial, too, but there are several arguments in favour of the significance of the observed effects. First, injections of the oily medium in which cord factor was dissolved were without effect, and the activity of cord factor was the same when injected in mineral and in vegetable oil. Second, there were no indications that the mice died from toxic manifestations rather than from infection. Gross haemorrhages of lungs and liver which occur after repeated cord factor injections were absent. The mice died with the symptoms of acute tuberculosis and the gross pathological findings were in agreement with what is usually found in heavily infected animals dying after similar intervals of time. The fact that under the combination effect the mice really die of a more severe type of tuberculosis than the non-injected control animals is further brought out by the bacterial counts in various organs. Thus it seems safe to conclude that under the influence of cord factor, experimental

tuberculosis takes a more acute course than it would otherwise by rendering the local tissues more suitable to support the growth of tubercle bacilli.

The specificity of the enhancing action of cord factor on infection is indicated by the failure of related lipid constituents of the tubercle bacillus to act likewise. The so-called lipopolysaccharide or wax D which is similar to Choucroun's " Pmko " (Asselineau, Choucroun and Lederer, 1950), mycolic acid of various strains, and a non-toxic fraction structurally similar to cord factor (Noll and Bloch, 1955) did not enhance tuberculous infections. On the other hand, cord factor failed to influence infections caused by staphylococci, streptococci or pneumococci.

There are several considerations leading us to believe that cord factor is released by the tubercle bacillus *in vivo*: the compound accumulates at the bacterial surface, whence it can be extracted without killing the bacteria; only with young virulent cultures does this surface extraction yield cord factor; under appropriate conditions *in vitro*, cord factor is released into the surrounding medium. It seems reasonable to conclude, therefore, that combination effects similar to the ones observed when injecting cord factor and tubercle bacilli might naturally occur in tuberculosis at organ sites where virulent organisms multiply in tissues.

The isoniazid experiments reported in the present paper revealed an antagonistic relationship between isoniazid and cord factor which appears to be quantitative. The possibility exists that this is a specific competitive inhibition, particularly since cord factor was ineffective in similarly inhibiting the action of other chemotherapeutic agents (Bloch, unpublished work). The failure of cord factor to interfere with the bacteriostatic effect of isoniazid *in vitro* might be due to the complete insolubility of cord factor in an aqueous culture fluid. It is also possible that the antagonistic effect observed *in vivo* may be an indirect one. Cord factor produces haemorrhagic areas suitable for bacterial multiplication. Since haemin has been shown to antagonise the action of isoniazid (Fisher, 1954), the accumulation of this blood constituent in these areas might account for the relative therapeutic ineffectiveness of isoniazid in cord factor-enhanced infections.

The good correlation previously found to exist between the production of cord factor and the virulence of tubercle bacilli, the specific infection-enhancing action of cord factor shown in the present experiments, and the antagonistic effect of cord factor toward a specific antituberculosis drug, all justify the previously suggested hypothesis that cord factor plays an important part in the pathogenicity of the tubercle bacillus.

SUMMARY.

Experiments are described in which the effects of cord factor on the course of tuberculosis in mice were studied. A single injection of cord factor, which by itself has no lasting damaging effects, causes tuberculous infections to progress more rapidly than they would otherwise. Both acute and chronic infections are significantly enhanced. Mice receiving an injection of cord factor prior to infection die sooner than controls. If such mice are sacrificed at various time intervals after infection, their lungs, livers and spleens contain more viable tubercle bacilli than the organs of control animals.

The enhancing effect of cord factor on murine tuberculosis is specific. Injections of mycolic acid or bacterial wax fractions other than cord factor fail to

influence the course of experimental tuberculosis. Staphylococcal, streptococcal and pneumococcal infections in mice are not affected by injections of cord factor.

Tuberculous infections enhanced by preceding injections of cord factor respond to a much lesser degree to the therapeutic action of isoniazid. The antagonism between the drug and cord factor is observed *in vivo* only. It is quantitative in nature, depending both on the amount of cord factor injected and on the dosage of isoniazid.

Materials were kindly supplied by the following persons or firms : Meningococcus toxin by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York, N.Y. Thorotrast by Heyden Chemical Company, New York, N.Y. Isoniazid by E. R. Squibb and Sons, New York, N.Y. Triton A20 and other related agents by Dr. P. D'Arcy Hart, Medical Research Council, London, England. Pyribenzamine by Ciba Pharmaceutical Products Inc., Summit, N.J.

The competent technical assistance of Miss Josephine C. Brosseau is gratefully acknowledged.

REFERENCES.

Asselineau, J., Bloch, H. and Lederer, E.—(1953) *Amer. Rev. Tuberc.*, **67**, 853.—(1954) *Biochim. biophys. Acta*, **15**, 136.

Idem, Choucroun, N. and Lederer, E.—(1950) *Ibid.*, **5**, 197.

Bloch, H.—(1950*a*) *J. exp. Med.*, **91**, 197.—(1950*b*) *Ibid.*, **92**, 507.—(1953) *Annu. Rev. Microbiol.*, **7**, 19.

Idem and Noll, H.—(1953*a*) *J. exp. Med.*, **97**, 1.—(1953*b*) *Trans. nat. Tuberc. Ass., N.Y.*, **49**, 94.

Idem, Sorkin, E. and Erlenmeyer, H.—(1953) *Amer. Rev. Tuberc.*, **67**, 629.

Cornforth, J. W., Hart, P. D'A., Rees, R. J. W. and Stock, J. A.—(1951) *Nature, Lond.*, **168**, 150.

Dubos, R. J. and Middlebrook, G.—(1947) *Amer. Rev. Tuberc.*, **56**, 334.

Fenner, F. and Leach, R. L.—(1953) *Ibid.*, **68**, 321.

Fisher, M. W.—(1954) *Ibid.*, **69**, 469.

Good, R. A. and Thomas, L.—(1952) *J. exp. Med.*, **96**, 625.

Hart, P. D'A. and Rees, R. J. W.—(1950) *Lancet*, ii, 391.

Middlebrook, G., Dubos, R. J. and Pierce, C. H.—(1947) *J. exp. Med.*, **86**, 175.

Noll, H.—(1953) *Proc. 6th Congr. int. Microbiol.*, in press.

Idem and Bloch, H.—(1953) *Amer. Rev. Tuberc.*, **67**, 828.—(1955) *J. biol. Chem.*, in press.

Stetson, C. A. and Good, R. A.—(1951) *J. exp. Med.*, **93**, 49.

PROPAGATION OF MYXOMA VIRUS IN ONE-DAY OLD MICE.

C. H. ANDREWES AND S. HARISIJADES.*
From the National Institute for Medical Research, Mill Hill, London, N.W.7.

Received for publication October 4, 1954.

THE virus of myxomatosis is considered to be specific to rabbits *sensu lato;* it produces a fatal disease in European rabbits (*Oryctolagus*) and local lesions in species of *Lepus* and the New World *Sylvilagus* (Aragao, 1943); also, it can be cultivated in fertile hen's eggs (Lush, 1937). The work now reported is the first to describe multiplication of this virus in a mammal other than a rabbit.

METHODS.

Passage in mice.—One-day-old mice of Webster's VSBS Rockefeller strain were inoculated intracerebrally with 0·01 ml. of passage material. Suspensions and dilutions were made in serum broth-saline (0·9 per cent NaCl containing 9 per cent of broth and 1 per cent horse serum). As a rule the same inoculum was given to all members of a litter except one which was marked and kept as an uninoculated control. For passage one or more mice were killed with chloroform, usually 4 or 5 days after inoculation; their brains were removed aseptically and ground in a TenBroeck grinder with serum broth-saline, 3 ml./brain. The suspension, after light spinning, was used to inoculate the next mice in the series. As a rule some mice of each litter were observed for 14 days before being discarded.

Titration in rabbits.—Brain suspensions and other fluids for titration were, after centrifugation, tested in falling tenfold dilutions in skins of the closely clipped flanks of male Dutch rabbits; 0·1 ml. of each dilution was injected intradermally. The local myxomatous lesions were measured daily, a final reading being taken as a rule on the fourth day; generalised pocks usually appeared by the fifth day, so that later readings were not possible.

Titration in eggs.—Three to five 11-day-old eggs were inoculated on the dropped chorio-allantoic membrane, each with 0·05 ml. of undiluted brain suspension. They were examined 3 days later, discrete pocks being counted with a lens; titre was expressed as the average number of pocks per inoculum. For very potent samples, dilution was necessary to produce membranes on which pocks could be counted.

RESULTS.

Passage in mice.

The myxoma virus could be propagated in series in infant mouse brains. Up to the present, thirty serial passages have been achieved.

The technique employed involves more than a hundredfold dilution at each passage and the titres lately obtained are as good as those in the first passage; so multiplication of the order of 10–60 times must have occurred. In the mice of the first passage series virus was recovered after 13 but not 20 days. Between the 3rd and 10th passages it was rarely obtained more than 6 days after inoculation; but at the 18th and 19th passes, virus was recovered 8 and 9 days after inoculation. The virus titre was usually maximal at 3 to 5 days; so this interval was chosen as best for successive passages. Fig. 1 shows the titres of brains of individual mice killed at various times after inoculation.

* In receipt of a W.H.O. Fellowship.

Supernatants of lightly centrifuged 5 per cent brain suspensions usually produced lesions in rabbit skins when diluted up to 10^{-3}; 10^{-4} dilutions were sometimes positive. The virus has produced no symptoms in the inoculated mice, which have continued to grow as well as the uninoculated controls. Spread of virus to such a control could not be demonstrated. The titre of virus in the mouse brains has shown no tendency to rise progressively as passages were continued. We were unsuccessful in attempting to propagate virus in brains of recently weaned (8–10 g.) or older mice. Mice of intermediate ages were not tested. In a single instance virus was recovered from the brain of a 20 g. mouse inoculated 3 days previously. No virus was recovered from liver and lung of one-day-old mice inoculated 3 days previously by the intraperitoneal and intranasal routes respectively.

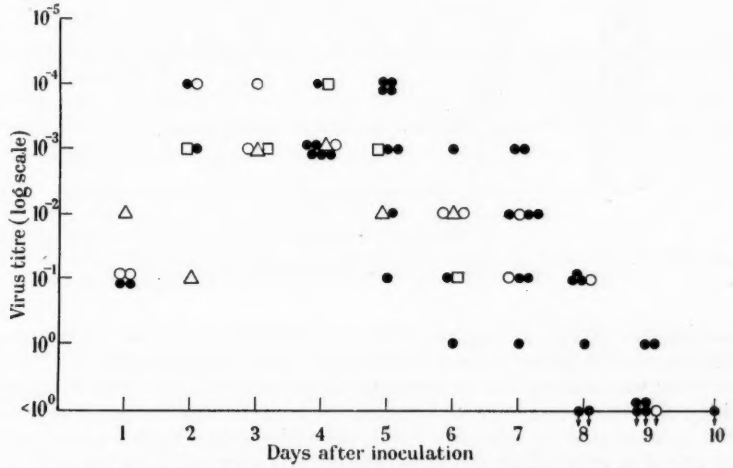


FIG. 1.—Virus titres in brains of mice killed at various times after inoculation.

○ △ □ Titres of individual mouse brains from 3 separate litters

● Titres of brains in all other experiments.

Difficulties encountered.

Serial passage of virus in mouse brains was not accomplished without setbacks. On numerous occasions, particularly around the tenth passage, virus fell in titre in successive passages and was finally lost. Brain suspensions of each passage were stored in a refrigerator at $-65°$; so it was always possible, after such an occurrence, to go back to stored material and carry on the sequence anew. We found, on several occasions, that when the virus was growing poorly the brain of one mouse might yield virus while that of a litter-mate was negative. Possibly we were, in such circumstances, working near the ID_{50} (50 per cent infective dose) of the virus. We noted, further, that even when things were going well, the harvest of virus was poor or erratic when mice were inoculated with diluted (10^{-2} or 10^{-3}) virus; moreover successive passages from such mice again yielded low titre virus.

It seemed possible that virus might only be multiplying just enough to compensate for the inevitable dilution which occurred at each transfer, so that a build-up in titre was hard to accomplish. Accordingly, we attempted to improve matters by concentrating virus tenfold before passing it. Supernatants of lightly spun suspensions were centrifuged for 30 min. at 8000 *g.* in an angle head centrifuge and deposits re-suspended in one-tenth the original volume of broth-serum-saline. Titration in rabbits' skins showed that tenfold concentration of virus was regularly achieved in this way. Even so, after carrying out such concentration at each of five successive passages we did not succeed in obtaining virus of unusually high titre. Nor did we attain better results by means of alternate passage (zig-zag passes) between mice and eggs ; 4 mouse- and 3 egg passes were included in this series.

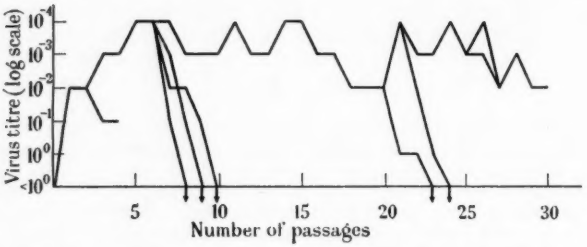


FIG. 2.—Variations in virus titre at different stages of passage.

Examination of Fig. 2 suggests that our difficulty arose partly because occasional litters of mice failed to permit adequate virus multiplication in their brains, so that virus was almost lost or brought down to low titre. We considered the possibility that on passage we had picked up a latent virus capable of interfering with the propagation of myxoma. We therefore mixed active virus with brain suspensions of mice in which the virus was doing badly and injected such mixtures either into brains of more infant mice or intradermally into rabbits. No regular inhibition of, or interference with the growth of virus could be demonstrated.

We obtained generally similar results, using mice of two other stocks—Parkes mice bred in this Institute and mice from the State Serum Institute, Copenhagen. The latter had been freed from infection with TO virus (mouse encephalomyelitis) by Dr. H. von Magnus. Nor could it be maintained that loss of virus titre was only apparent and that a mutant of low pathogenicity for rabbits had emerged. When virus was obtained having such low titre in rabbits, the ratio rabbit/egg titre did not appreciably change. Further, rabbits failing to respond after injection of mouse brain suspensions did not show resistance to later challenge with active myxoma.

Results in eggs.

In general virus titres in eggs were proportional to those in the rabbit ; we did not encounter, even after 35 egg-to-egg passages, samples with high egg- and low rabbit-titre such as would be expected with modification of the virus (*cf.* Haagen and Dscheng-Hsing, 1938).

In conformity with the results in mice, we found that titres of infected mouse brains tested on eggs were at times anomalous: single virus suspensions produced, on some occasions, plentiful pocks on one chorio-allantoic membrane, none at all on another.

Behaviour of mouse-passage virus in rabbits.

After thirty passes the myxoma virus was little if at all altered in its virulence for rabbits. One rabbit receiving diluted virus of the 17th mouse passage and another at the 25th pass, underwent a typical infection but recovered. Another (10th pass virus) survived for 19 days. All others died of typical myxomatosis after 7 to 14 days (10–12 days in 26/35 rabbits).

DISCUSSION AND SUMMARY.

The specificity of myxoma for rabbits and their close relatives is not absolute; thirty serial passages in brains of new-born mice have been achieved. Virus so passed has not acquired pathogenicity for the mouse nor lost it for the rabbit. This work gives no grounds for believing that the virus will infect species other than the rabbit in nature.

REFERENCES.

ARAGAO, H. DE B.—(1943) *Mem. Inst. Osw. Cruz*, **38**, 93.
HAAGEN, E. AND DSCHENG-HSING, D.—(1938) *Zbl. Bakt.* (1 Abt. Orig.), **143**, 23.
LUSH, D.—(1937) *Aust. J. exp. Biol. med. Sci.*, **15**, 131.

RETENTION AND EXCRETION OF ^{90}Sr BY ADULT RABBITS.*

JENIFER JOWSEY, MAUREEN OWEN†, MARGARET TUTT†
AND
JANET VAUGHAN.

From the Nuffield Department of Medicine and the Nuffield Institute for Medical Research, Oxford.

Received for publication October 21, 1954.

DETAILED studies of retention and excretion of strontium isotopes, following a single intravenous injection, by rabbits 1 day, 5–7 weeks and 10–11 months old have already been reported (Kidman, Tutt and Vaughan, 1950; Tutt, Kidman, Rayner and Vaughan, 1952; Kidman, Rayner, Tutt and Vaughan, 1952; Jowsey, Rayner, Tutt and Vaughan, 1953). It was noted (Kidman *et al.*, 1952) that in one animal 3½ years old killed nine days after injection, there appeared to be areas of localised concentration of strontium in the bone. Investigation of daily excretion of ^{90}Sr in animals several months after injection also showed a marked irregularity. A more detailed study of the behaviour of ^{90}Sr in rabbits 1 year or more old at the time of injection was therefore undertaken.

EXPERIMENTAL.

Rabbits were of the same stock and kept on the same diet as those used in previous experiments. Nine rabbits, 12 months old, were given a single intravenous injection of $^{90}SrCl_2$ in carrier-free solution (80 μc. per kg. body wt.). They were killed at intervals up to 18 months later. One rabbit, 7½ years old, was given a single intravenous injection of 1000 μc./kg. $^{90}SrCl_2$ and killed 24 hr. later. The whole skeleton was removed. One tibia and one femur from all rabbits were used for the preparation of macroscopic autoradiographs. The other tibia from the oldest rabbit was used to prepare cross sections for microscopic autoradiography and histological examination. The retention of strontium in the rest of the skeleton was estimated; a calculated amount was added for the ^{90}Sr present in the bones used for autoradiography. To estimate daily excretion two of the nine rabbits whose bones were used for autoradiography were placed in separate metabolism cages 4 weeks before they were killed at 12 months after injection. New cages were used at the start of the period and new collecting bottles were used daily. Cages were washed down and the activity in the washings, which was estimated separately, was added to that in the urine.

Estimation of ^{90}Sr.—Estimation of ^{90}Sr was carried out as described for ^{89}Sr except that all samples were left for 20 days to allow restoration of equilibrium (Tutt *et al.*, 1952). The urine activity was extremely low and counts were made over at least 1 hr.

Microradiographs and autoradiographs.—(i) Macroscopic autoradiographs were prepared as previously described (Kidman *et al.*, 1952; Jowsey, Rayner, Tutt and Vaughan, 1953). (ii) Microradiographs and microscopic autoradiographs were prepared from the same sections by methods previously described (Jowsey, Owen and Vaughan, 1953).

Histological sections.—The lower half of the tibia was sawn into three pieces and placed in a solution containing sodium salts of ethylene-diamine-tetraacetic acid for decalcification, the pH of the solution being adjusted to 7·5. In some instances sections were decalcified

* This work was begun on behalf of the Protection Sub-Committee of the Medical Research Council's Committee on Medical and Biological Applications of Nuclear Physics.

† Nuffield Graduate Assistant.

after microradiography and autoradiography, the stripping film being first removed. Sections were stained with eosin azure and by the periodic acid-Schiff technique (Pearse, 1953).

RESULTS.

Retention in Animals 12 months old at Time of Injection.

The total retention in the skeleton 9 days after injection was 12·7 per cent of the injected dose. Six months after injection it had fallen to 4·3 per cent $\pm$ 0·72. Twelve months after injection it was 2·1 per cent $\pm$ 0·35 and eighteen months after injection it was 2·2 per cent.

Excretion.

The figures for the daily excretion of ^{90}Sr in the urine in one rabbit killed 12 months after injection varied in the month preceding death from 0·0007 per cent to 0·0147 per cent of the injected dose with a mean figure of 0·0031 $\pm$ 0·0008. In a second rabbit the mean figure was 0·0032 $\pm$ 0·0003 ranging from 0·0010 per cent to 0·0061 per cent.

Macroscopic Autoradiographs from Rabbits 1 year old and 7½ years old.

Autoradiographs prepared from longitudinal sections of bones removed at all times after injection irrespective of dose or age showed an irregular patchy concentration of ^{90}Sr in the epiphysis, in endosteal bone, periosteal bone and throughout cortical bone. The areas of concentration appeared more frequently in endosteal than in periosteal bone. There was a faint diffuse reaction throughout mineralized bone, most noticeable in the animal given the high dose (Fig. 1, 2).

Microautoradiographs of Cross Sections of the Tibia of a Rabbit 7½ years old.

The bone of the rabbit aged 7½ years proved brittle and difficult to grind without breaknig. The cracks were easily distinguishable in the microradiographs as artefacts. The bone was characterised by a high degree of calcification and by the presence of few building sites and Haversian canals (Fig. 3). In some sections there was a defined area of endosteal bone which was poorly calcified (Fig. 4). Only occasional small areas of periosteal bone of low calcification were seen in any of the twelve microradiographs examined. Since the macroscopic autoradiographs showed patches of concentrated ^{90}Sr in the periosteum, it is probable that periosteal bone was torn away in the grinding process. The building sites showed varying degrees of calcification in the surrounding bone, but compared with the bones of younger animals the zone of low calcification around active building sites was narrow. Short irregular lines of extremely highly calcified tissue—" silver lines "—appeared in the middle of the corticalis in some sections (Fig. 4).

Microautoradiographs of Cross Sections of the Tibia of a Rabbit 7½ years old.

There was marked concentration of reaction over areas of low calcification around some of the building sites (Fig. 5, 6). There was slight reaction over parts

of lowly calcified endosteal bone. Over the highly calcified cortical bone there was a slight reaction recognisable only when the actual grains reacting were counted both in background and over bone.

Histological Sections.

Detailed histological studies are not for discussion here, but it is significant that in the serial histological sections taken from the lower third of the tibia a strongly positive periodic acid-Schiff reaction (Pearse, 1953) was given by small areas of bone similar in shape and distribution to the " silver lines " seen in microradiographs. Such areas also stained cherry red with eosin azure. A direct comparison of a microradiograph showing " silver lines " with the section from which it was prepared subsequently decalcified and stained, confirmed that the areas showing the positive periodic acid-Schiff reaction were identical with the highly calcified silver lines. It appears justifiable to conclude therefore that the silver lines are remnants of calcified cartilage with a high mucopolysaccharide content.

DISCUSSION.

The pattern of deposition of ^{90}Sr in the bone of fully grown rabbits as demonstrated by autoradiographs resembles that in fully grown monkeys (Jowsey, Owen and Vaughan, 1953) rather than that in rabbits given ^{90}Sr before they are fully grown (Kidman *et al.*, 1952 ; Jowsey, Rayner, Tutt and Vaughan, 1953). ^{90}Sr in both adult monkeys and rabbits is concentrated unevenly in cortical bone, particularly in active building sites and to a lesser extent in areas of lowly calcified endosteal and periosteal bone throughout the shaft, while in young growing rabbits and monkeys it is found beneath the endosteum in the region of the metaphysis, in the epiphyseal plate and trabeculae, and beneath the periosteum in the middle of the shaft.

In both the old and the young, ^{90}Sr is concentrated in areas of active bone growth, but since the sites of activity vary with age, the sites of concentration vary too. The proportion of actively growing bone to less active or more stable bone is greater in the young than in the old. Rabbits aged 5–7 weeks at the time

---

DESCRIPTION OF PLATES.

Macroscopic Autoradiographs Prepared from Longitudinal Sections.

FIG. 1.—Lower end of femur of rabbit 12 months old injected ^{90}Sr (80 μc./kg.) killed 6 months later. Patchy distribution of localised reaction in epiphysis, periosteal bone, cortical bone and endosteal bone. × 1½.

FIG. 2.—Shaft of tibia of rabbit aged 7½ years injected ^{90}Sr (1000 μc./kg.) killed 24 hr. later. Same patchy distribution as in Fig. 1. × 2.

Microradiographs Cross Section Upper Third Shaft of Tibia, Rabbit aged 7½ years Injected ^{90}Sr (1000 μc./kg.) Killed 24 hr. later.

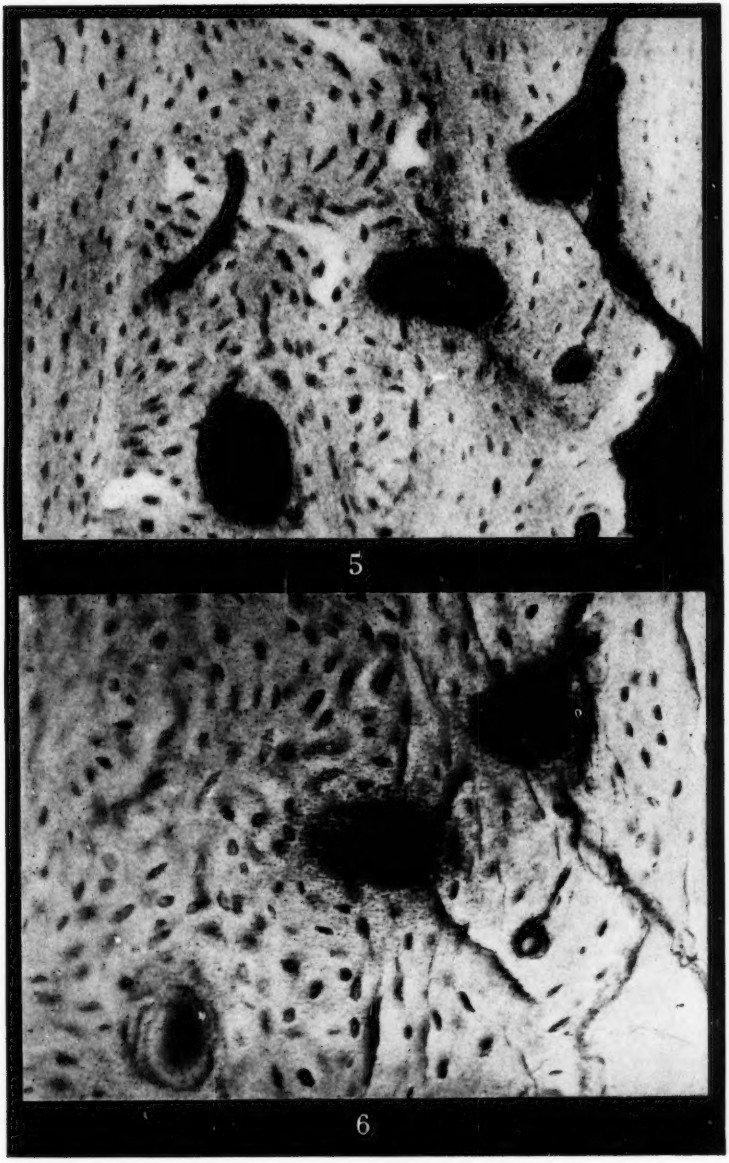
FIG. 3.—Note (1) high calcification ; (ii) small building sites with uneven areas of surrounding low calcification. × 30.

FIG. 4.—Note (i) small building sites showing low calcification of surrounding bone ; (ii) " silver lines " ; (iii) low calcification of endosteal bone. × 100.

FIG. 5.—Note (i) two building sites showing areas of low calcification and another with high calcification ; (ii) silver lines. × 200.

FIG. 6.—*Microscopic autoradiograph left on section from which Fig. 5 prepared.* Note (i) heavy localized reaction over areas of low calcification associated with two building sites ; (ii) slight reaction over space of third building site. × 200.

Jowsey, Owen, Tutt and Vaughan.



5

6

of injection retain 11·0 per cent of the injected dose 18 months later (Jowsey, Rayner, Tutt and Vaughan, 1953), while rabbits aged 12 months at the time of injection retain only 2·2 per cent of the injected dose. The fact that retention appears no lower at 18 months than at 12 months after injection in both young and old animals is probably due to the fact that the turnover of bone salt in older animals is extremely slow, and large numbers of animals would be required to demonstrate a significant difference, if it exists, at long periods after injection.

Though total retention in the old is much less than in the young, what is retained in both is highly concentrated. The strontium present in the skeleton of an old animal, though the total amount may be small, is concentrated in a few building sites active at the time of injection, and those areas are exposed to heavy radiation.

It is of importance from the point of view of radiation hazard that remnants of calcified cartilage may persist in the skeleton of a rabbit aged 7½ years. Growth in length of the long bones, dependent on endochondral calcification, ceases when the rabbit is about 6 months old, but resorption of all calcified cartilage remnants in the normal process of resorption and remodelling does not necessarily occur. It follows that if such calcified remnants had taken up a radioactive isotope in the first six months of life, they would have continued to be an area of concentrated radioactivity within the bone throughout life.

The occurrence of only a few scattered areas of heavy concentration of ^{90}Sr may account for the apparent uneven excretion from day to day of this isotope. The mean values for daily excretion over a period of a month in two rabbits one year after injection was remarkably alike, 0·0031 per cent of the injected dose and 0·0032 per cent of the injected dose, though the daily variation was considerable. Apposition and resorption go on even in old animals, but the present autoradiographic study shows that these processes occur only in localised and scattered sites at any one time. Resorption of an area of bone where in the past ^{90}Sr has been laid down would result in high excretion, but such areas are few and resorption may well occur for some days or weeks in areas of bone containing no ^{90}Sr. At such time excretion would be negligible. On the other hand, when such small quantities of isotope are involved the uneven excretion might be attributed to difficulties involved in accurate collection. ^{90}Sr is readily absorbed on surfaces. Care was taken to use fresh glassware daily and to wash down the metabolism cages and glassware with acid solution containing carrier in order to remove the ^{90}Sr if present. Whatever the explanation, it is clear that excreta must be analysed over long periods if a correct figure for excretion rate is needed.

SUMMARY.

Observations on the retention, excretion and distribution of ^{90}Sr in the skeleton of rabbits aged 1 year at the time of injection and killed at intervals up to 18 months later, are recorded, together with a microscopic study of the distribution of the same isotope in the tibia of a rabbit 7½ years old at the time of injection.

The distribution of ^{90}Sr in tibias of the adult rabbits resembles that in the tibias of adult monkeys rather than that in the tibias of growing rabbits.

In old animals ^{90}Sr is retained in areas of active bone growth, *i.e.*, in scattered building sites in cortical bone and to a lesser extent in areas of low calcification in the endosteal bone and periosteal bone.

Excretion of ^{90}Sr in old animals long after injection appears to be irregular from day to day.

Though the total amount of strontium retained in old animals is much less than that in young animals, what is present is highly concentrated and therefore the risk of radiation injury is serious.

One of us (J. V.) has had a personal grant from the Medical Research Council.

REFERENCES.

JOWSEY, JENIFER, OWEN, MAUREEN AND VAUGHAN, JANET M.—(1953) *Brit. J. exp. Path.*, **34**, 661.

Eadem, RAYNER, BARBARA, TUTT, MARGARET L. AND VAUGHAN, JANET M.—(1953) *Ibid.*, **34**, 384.

KIDMAN, BARBARA, RAYNER, BARBARA, TUTT, MARGARET L. AND VAUGHAN, JANET M.—(1952) *J. Path. Bact.*, **64**, 453.

Eadem, TUTT, MARGARET L. AND VAUGHAN, JANET M.—(1950) *Ibid.*, **62**, 209.

PEARSE, A. G. E.—(1953) ' Histochemistry Theoretical and Applied.' London (J. & A. Churchill Ltd.), p. 431.

TUTT, MARGARET L., KIDMAN, BARBARA, RAYNER, BARBARA AND VAUGHAN, JANET M.—(1952) *Brit. J. exp. Path.*, **33**, 207.

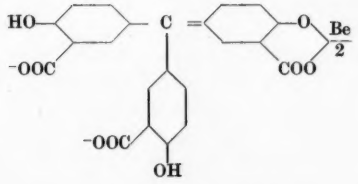
THE MODIFICATION OF BERYLLIUM-INDUCED TISSUE DAMAGE IN MICE BY THERAPY WITH AURINTRICARBOXYLIC ACID.

H. LISCO AND MARCIA R. WHITE.

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois.

Received for publication October 27, 1954.

IT has recently been shown that the effects of a lethal dose of beryllium in mice can be almost completely abolished by the administration of a single dose of aurintricarboxylic acid (ATA) (White, Finkel and Schubert, 1951). Intravenous injection of this dye provides protection to 90 per cent of animals poisoned with a dose of beryllium that would otherwise result in death in 2–5 days. It has been shown subsequently that protection is not caused by increased elimination or by redistribution of beryllium, but rather by inactivation *in situ*. Inactivation is accomplished by the formation of a stable and non-toxic lake between the dye aurintricarboxylic acid and the ionised beryllium. The first stage in the production of the lake involves the formation of the following chelate between the two substances :



Lake formation renders free beryllium ions incapable of combining with proteins and other cell constituents, and thus prevents tissue damage (Schubert, White and Lindenbaum, 1952).

Although survival data are convincing proof of therapeutic efficacy, they do not by themselves disclose information on the manner in which tissue damage is prevented or modified. The present experiments were undertaken to study the morphological events associated with the remarkable therapeutic effect of this dye. To this end the sequences of tissue damage were examined in beryllium-poisoned mice with and without therapy with aurintricarboxylic acid.

MATERIALS AND METHODS.

The experimental procedures were designed to parallel as closely as possible the earlier survival studies (White *et al.*, 1951). Two separate experiments were performed, the second almost an exact duplicate of the first. Young adult CF1 female mice were divided at random into three groups, receiving : (1) beryllium only (" untreated mice ") ; (2) beryllium and ATA (" treated animals ") ; (3) ATA only. Uninjected animals served as controls. The beryllium

was injected intravenously as an aqueous solution of beryllium sulfate, containing 0·07 mg./Be/ml. at pH ~ 3·5. Each mouse received 0·7 mg. Be/kg. (~ 17 μg. Be per mouse), a dose that causes death of 98 per cent of the animals in 3–5 days (White and Schubert, 1954). The ammonium salt of ATA, Eastman practical grade (440 mg./kg. = LD_{50}/21 days in mice), was used in a freshly prepared aqueous unsterilised solution; the pH was adjusted to 7·3 with dilute NaOH. Each mouse was injected intravenously with 4 mg. (~ 180 mg./kg.) 1 hr. after beryllium administration. In each experiment four or more animals from each group were sacrificed at random by cervical fracture at intervals from 12 hr. to 14 days and at 320 days. The spleen, kidney and four different lobes of the liver were fixed immediately in Helly's fluid in the first experiment. In the second experiment all tissues were fixed in 10 per cent formalin. Femora and a section of the vertebral column were decalcified in a solution of 3 per cent HNO_3. All tissues were embedded in paraffin and the sections stained with haematoxylin and eosin; no special stains were used.

RESULTS.

Necrotising lesions identical with those described by Scott (1948) and Aldridge, Barnes and Denz (1949) have been observed in liver, spleen, kidney and, in addition, in the bone-marrow. The discussion of the effect of ATA upon these lesions will proceed in the order in which it is summarised in Table I. The results of the two experiments are tabulated separately as indicated by a double spacing. The animals are arranged in the same random distribution in which they were chosen for sacrifice, and from organ to organ the arrangement of the animals is identical.

Animals receiving beryllium only became sick 24–48 hr. after injection. Some died before the second day and very few survived until the third day. The mice sacrificed at 72 hr. were thus a very select group and all were moribund. All mice injected with beryllium and ATA were healthy in appearance until sacrificed.

Liver.

Liver damage in animals receiving beryllium only increased markedly with time, terminating in severe and widespread necrosis of liver parenchyma. ATA-treated mice showed considerably less damage (Fig. 1, 2).

In the former, alterations at 12 hr. were characterised by swelling of liver cells associated with increased eosinophilia of the cytoplasm; there were a few dead liver cells and conspicuous numbers of polymorphonuclear leucocytes. Identical though less conspicuous lesions were noted in the ATA-treated animals at this time. The 24-hr. specimens showed appreciable differences between the two groups, namely an exacerbation of damage in all untreated animals and only a moderate increase in the extent of the lesions in the treated mice. Further spread of liver damage was evident in all untreated animals at 48 hr., so that very little viable parenchyma remained. In contrast to this picture of almost universal cell death in the untreated mice, the ATA-treated group at this time was free of such lesions with the exception of one animal that exhibited advanced focal necroses.

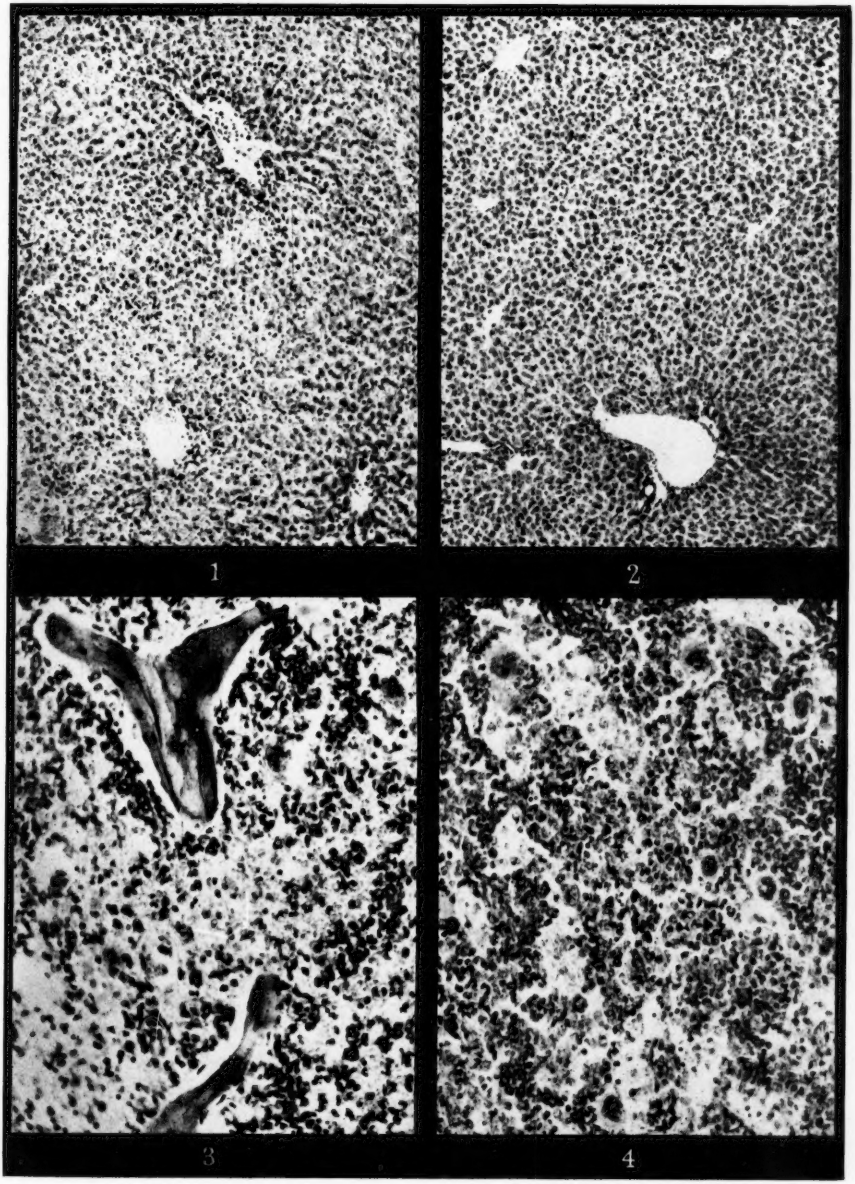
EXPLANATION OF PLATES.

FIG. 1.—Widespread (++++) destruction of liver tissue at 48 hr. (beryllium alone). × 95.

FIG. 2.—Liver of mouse injected with beryllium and treated with ATA, sacrificed at 48 hr. × 95.

FIG. 3.—Bone marrow from metaphysis of femur of animal injected with beryllium, sacrificed at 48 hr., showing extreme damage (++++). × 235.

FIG. 4.—Bone-marrow from animal injected with beryllium, treated with ATA, and sacrificed at 48 hr. Recovery from damage and " shift to the right " in myelopoiesis. Occasional necrotic cells (+ damage). × 235.



Lisco and White.

TABLE I.—*Comparison of Degree of Beryllium Damage in Treated and Untreated Mice.*

Tissue.	12 hours. Be.	12 hours. Be + ATA.	24 hours. Be.	24 hours. Be + ATA.	48 hours.* Be.	48 hours.* Be + ATA.	72 hours. Be.†	72 hours. Be + ATA.	7 days. Be + ATA.	14 days. Be + ATA.	320 days. Be + ‡ ATA.
Liver	+	+	++	+	++++	0	$+^{R}$	0	0	0	0
	+	+	++	+	++++	0	$++^{R}$	0	0	0	0
	+	+	+++	+	++++	0	..	0	0	0	0
	+	+	++	++	++++	0	..	0	0	0	0
	..	..	++	..	++++	0	..	0	..	..	0
	+	+	++	+	++++	0	$++^{R}$	+	..	0	..
	+	+	++	++	++++	0	$++^{R}$	0	..	0	..
	+	+	++	+	++++	$+++^{R}$	$+++^{R}$	0	..	0	..
	+	+	++	++	++++	0	$+++^{R}$	0	..	0	..
Spleen	++	+	+++	++	++++	+	0	0	0	0	0
	++	+	++	++	++++	+	++	0	0	0	0
	++	++	+++	++	++++	+	..	0	0	0	0
	++	+	+++	++	++++	+	..	0	0	0	0
	..	..	+++	..	++++	+	..	0	..	..	0
	..	+	++	++	+++	+	++	0	..	0	..
	+	+	+	++	+++	+	0	0	..	0	..
	+	+	++	++	+++	+	++	0	..	0	..
	++	++	++	++	+++	+	++	0	..	0	..
Bone-marrow	+	+	++	+	++++	+++	++++	0	..	0	0
	+	+	++	+	++++	+	++++	0	..	0	0
	0	+	+++	+	++++	+	++++	0	..	0	0
	+	+	++	+	++++	+	++++	0	..	0	0
											0
Kidney	0	+	0	++	+	$+^{R}$	0	$++^{R}$	0	0	0
	0	0	..	+++	++	$++^{R}$	0	$+++^{R}$	0	0	0
	0	+++	+	++	+	$+^{R}$	..	$++^{R}$	0	0	0
	0	+	0	0	+	+	..	$+^{R}$	0	0	0
	..	..	0	..	0	$++^{R}$	..	0	..	..	0
	0	0	0	+	0	0	0	0	..	0	..
	0	0	0	++	0	0	0	$++++^{R}$	..	0	..
	0	0	0	+	0	+	0	0	..	0	..
	0	0	0	+	0	+	0	+	..	0	..

* The symbols $+^{R}$ to $++++^{R}$ signify extent of beryllium damage which is under repair.
† All animals moribund. A select group.
‡ Ten mice were sacrificed at 320 days.

At 72 hr. the livers of the few survivors receiving only beryllium showed a mixture of injury and repair, while those of ATA-treated animals had no evidence of beryllium damage with the exception of two animals that had several isolated and incompletely healed focal necroses. The livers of ATA-treated animals sacrificed at 7 and 14 days were free of beryllium damage, although 2 of the 12 animals showed several small well-circumscribed subcapsular necroses in the process of resorption and healing, and one showed subcapsular accumulations of pigment-laden cells in an otherwise intact liver. Ten ATA-treated animals sacrificed at 320 days had no lesions that could be attributed to the presence of beryllium.

Most of the animals receiving ATA, alone or in combination with beryllium, showed minor but distinct irregularities of liver cells. These consisted primarily of a depletion of the protoplasm, of vacuoles (fat?) in the cytoplasm, and also of a peculiar tinctorial distinctiveness of the walls of many cells which stained intensely with eosin. Variations in size and shape of liver cells obscured the regular structure of the liver lobules. These irregularities, almost uniformly present by 48 hr., persisted for at least 14 days. This appearance of the liver was in contrast to that of young control animals, but it was not distinguishable from that of old control mice in this and other experiments. The significance of these cellular variations is thus not clear, but it should be emphasised that the livers of ATA-treated animals were free of beryllium damage beginning at 48 hr.

In summary then, ATA diminished liver damage in the early phase of beryllium poisoning and prevented further beryllium injury from occurring during a period of 320 days.

Spleen.

The spleen was found to be very vulnerable to beryllium. Appreciable damage developed in untreated and treated animals, but it clearly predominated in the former.

In untreated animals at 12 hr. lesions were seen in the white and red pulp. In both regions these were characterised by cytolytic changes and by the accumulation of cellular débris. The earliest evidence of injury was noted in phagocytes and other cells associated with the sinusoids of the perifollicular zones of the red pulp. This zone eventually became depleted of cells and appeared as a conspicuous pale halo, often marking the entire circumference of lymph follicles. With time the damage spread into the remainder of the red pulp and there was widespread destruction of cells at 24 hr. The focal aggregates of extramedullary myelo- and erythropoiesis were severely damaged and at 48 hr. the red pulp was almost entirely depleted of living cellular elements.

Simultaneously with the extension of damage into the red pulp from the perifollicular zone (24 hr.) there appeared striking evidence of destruction of cells and of phagocytosis of débris in the lymph follicles, but lymph follicles were never destroyed as completely as the red pulp. At the height of the damage (48 hr.) they remained as shrunken islands of living cells in an otherwise severely damaged spleen. Survivors at 72 hr. showed considerably less damage in both red and white pulp.

ATA-treated mice had identical lesions, but a reduction of the extent of injury was evident at all time intervals, beginning at 12 hr. Damage reached its peak at 24 hr. and at 48 hr. it was negligible. The red pulp as well as the white pulp

of many animals at 48 and 72 hr. was very cellular. However, no significant deviations from controls were noted at 7, 14 and 320 days.

ATA injected alone had no noticeable effect on the cells of the red or white pulp.

Bone-marrow.

Bone-marrow injury was present in both groups of animals. Untreated mice developed extensive lesions involving the entire marrow, whereas ATA-treated mice showed much less injury and eventually complete repair of the damage (Fig. 3, 4).

The lesions in untreated as well as ATA-treated animals at 12 hr. consisted of a mild hyperaemia and of minute foci of phagocytised débris. In the untreated animals injury at 24 hr. consisted primarily of hyperaemia, small haemorrhages and focal destruction of cells involving granulopoietic as well as erythropoietic elements. A remarkable intensification of damage occurred between 24 and 48 hr. in untreated mice. This led to widespread haemorrhages, large areas of necrosis, and cellular depletion so that only a few islands of viable haematopoietic tissue remained. The lesions involved even larger portions of the marrow at 3 days.

ATA-treated mice showed remarkably less injury to the bone-marrow. The peak of the mild damage was noted at 48 hr. and no lesions were seen at later intervals. No studies were made on the bone-marrow following injection of ATA alone.

Kidney.

Although renal damage was observed in both groups of animals, it was apparent that 4 mg. ATA, instead of ameliorating beryllium injury as it had done in other tissues, potentiated it.

The mild renal injury in mice receiving beryllium only occurred almost exclusively at 48 hr. It consisted of necrosis of tubular epithelium involving the distal portion of the proximal convoluted tubule of a few nephrons. There were no lesions and no evidence of repair in the kidneys of survivors at 72 hr.

The renal lesions of ATA-treated animals at 12 hr. were of the same character as those seen at 48 hr. in untreated mice. They had increased in severity and involved a greater number of tubules at 24 hr. Damaged glomeruli were noted in animals with massive necrosis of tubular epithelium. Animals at 24 hr. showed little, if any, evidence of regeneration in contrast to those at 48 hr., in which much repair was noted. At 72 hr. the majority of the animals exhibited tubular lesions in an advanced stage of repair, while animals sacrificed at 7, 14 and 320 days were indistinguishable from controls.

Study of the kidneys of animals receiving a single dose of 4 mg. ATA alone did not reveal any effects of this material on the tubular epithelium at any time interval.

Comparative therapeutic effectiveness of 2 mg. ATA.

In order to test the therapeutic effect of one-half the dose of ATA under otherwise identical experimental conditions, additional animals treated with 2 mg. ATA were studied at 48 and 72 hr. and at 320 days. The results showed that this

dose of ATA protected the livers and spleens of beryllium-poisoned animals as well as did 4 mg. (Table II).

Of particular interest was the fact that there was no intensification of injury in the kidneys, in contrast to the animals receiving 4 mg. ATA. The amount of kidney damage at 48 hr. was similar to that in animals receiving beryllium only and the kidneys of all animals sacrificed at 72 hr. and at 320 days were without demonstrable lesions.

TABLE II.—*Comparative Effectiveness of 2 and 4 mg. of ATA on Degree of Beryllium Damage.**

Tissue.	48 hours.			72 hours.		
	Be only.	Be + 2 mg. ATA.	Be + 4 mg. ATA.	Be only.	Be + 2 mg. ATA.	Be + 4 mg. ATA.
Liver	++++	0	0	$+^R$	0	0
	++++	+	0	$+^R$	0	0
	++++	0	0	..	0	0
	++++	0	0	..	0	0
	++++	0	0	..	0	0
		+	..	..	0	..
Spleen	++++	+	+	0	+	0
	++++	++	+	++	0	0
	++++	+	+	..	0	0
	++++	+	+	..	0	0
	++++	+	+	..	0	0
		+	..	..	0	..
Kidney	+	0	$+^R$	0	0	$++^R$
	++	+	$++^R$	0	0	$+++^R$
	+	$++^R$	$+^R$	..	0	$+++^R$
	+	0	+	..	$+^R$	$+^R$
	0	++	$++^R$	..	0	0
		+	..	..	0	..

* For explanation of symbols see Table I.

DISCUSSION.

Previous experiments on the therapeutic effects of aurintricarboxylic acid in acute beryllium poisoning in mice had strongly suggested that this dye acted by preventing tissue damage. The present studies have confirmed this and have made it apparent that the protective action was due to a reduction of the toxicity of beryllium in the tissues, which led to a considerable diminution of injury in the liver, spleen and bone-marrow.

At the time when ATA was injected into these animals, approximately one-half of the beryllium still remained in the blood, so that inactivation of beryllium by ATA must have taken place in the blood stream as well as in the tissues. It is apparent that the injury which was seen in the ATA-treated mice was caused primarily within the first hour. The injury initiated during this time was mild and it was repaired rapidly. The absence of additional fresh lesions in liver, spleen and bone-marrow after 24 hr. is evidence for the speed of inactivation of beryllium. Inactivation is also permanent, as judged by the absence of tissue injury referable to beryllium at 320 days.

In contrast to the protection of these organs, kidney damage was not diminished in ATA-treated animals and, with a dose of 4 mg. of ATA, there was, in fact, intensification of tubular injury. Several factors may have contributed to the causation of this unexpected finding. A threefold increase of beryllium in the kidneys has been shown to follow administration of this dose of ATA in mice (Schubert *et al.*, 1952) and this may have been responsible for the added damage, even though there was more than enough ATA present to inactivate the beryllium. However, the conditions prevailing in the kidneys, especially the low pH in the renal tubules, may very well have decreased the stability of the Be-ATA complex. For example, it has been shown by Meek and Banks (1951) that a decrease in pH decreases the stability of the chelate of beryllium with sulfosalicylate. It is also known that a low pH increases the solubility of beryllium (Schubert and White, 1950). The failure of the 2 mg. dose of ATA to potentiate kidney damage may be ascribed to the fact that this dose probably did not shift additional beryllium to the kidneys.

Regardless of the exact mechanisms involved in the pathogenesis of the renal lesions in ATA-treated animals, it should be emphasised that the tubular damage was transient and that no permanent lesions resulted. The kidneys of all animals at 7, 14 and 320 days were normal.

The present work has again demonstrated the extremely toxic properties of beryllium to diverse cells and tissues of rodents. In amplification of earlier studies, extensive injury has been found in the white pulp of the spleen, demonstrating the great susceptibility of lymphocytes to beryllium. Furthermore, it was found that bone-marrow was as vulnerable as liver and spleen to a lethal dose of beryllium. It is evident, therefore, that death of animals in acute beryllium poisoning is the result of cumulative and progressive injury to a number of organs.

Since in the present experiments the disposition of beryllium in liver, spleen and bone-marrow was typical of colloidal particles it is likely that much of the material was initially taken up by the phagocytic cells of the reticulo-endothelial system. Examination of the early and mild lesions in the spleens showed that phagocytes were indeed involved in the uptake of beryllium, for many of them, located in the perifollicular zones of the red pulp, contained granules of pink-staining and homogenous material in the cytoplasm and they showed much evidence of irritation, especially in the appearance of the nuclei ; many of them were necrotic. The participation of the reticulo-endothelial system in the removal of beryllium compounds from the blood has been pointed out by Aldridge *et al.* (1949), who described the similarity in the gross distribution of beryllium compounds of this type and of India ink.

In contrast to ethylenediamine tetraacetic acid (EDTA) and to 2,3-dimercaptopropanol (BAL), which are chelating agents used in the treatment of heavy metal poisoning and which act by promoting the excretion of the metal, ATA causes no increase in excretion of beryllium. Rather it acts *in situ* by the formation of insoluble lakes with ionised beryllium in the tissues, thereby rendering the beryllium non-toxic. Thus, while lake formation includes chelation, the mode of action of ATA differs from that of EDTA and BAL, and the present observations on the sequences of tissue injury and repair in beryllium poisoning as modified by ATA illustrate a different principle of pharmacological action, namely the inactivation of a toxic metal by lake formation without concomitant excretion.

SUMMARY.

A comparative morphological study has been made of the sequences of tissue damage in experimental beryllium poisoning in mice, with and without therapy with aurintricarboxylic acid (ATA).

A single injection of ATA greatly diminished beryllium injury in the liver, spleen and bone-marrow of mice poisoned with a LD_{98}/3 days. These organs were free of any trace of beryllium damage at 3 days and 320 days after injection.

The mild kidney damage which is caused when beryllium is given alone was potentiated in mice receiving 4 mg. of ATA, although the renal injury was transient and the kidneys were normal after seven days. No potentiation of kidney damage was seen in mice treated with only 2 mg. of ATA.

The authors are indebted to Olive C. Kimball for technical assistance and to Jane K. Glaser for valuable aid in the preparation of the photomicrographs. Discussions with Dr. J. Schubert throughout this investigation have been very helpful.

REFERENCES.

ALDRIDGE, W. N., BARNES, J. M. AND DENZ, F. A.—(1949) *Brit. J. exp. Path.*, **30**, 375.
MEEK, H. V. AND BANKS, C. V.—(1951) *J. Amer. chem. Soc.*, **73**, 4108.
SCHUBERT, J. AND WHITE, M. R.—(1950) *J. Lab. clin. Med.*, **35**, 854.
Iidem AND LINDENBAUM, A.—(1952) *J. biol. Chem.*, **196**, 279.
SCOTT, J. K.—(1948) *Arch. Path.*, **45**, 354.
WHITE, M. R., FINKEL, A. J. AND SCHUBERT, J.—(1951) *J. Pharmacol.*, **102**, 88.
Idem AND SCHUBERT, J.—(1954) *Arch. Biochem. Biophys.*, **52**, 133.

GLYCOGEN ACCUMULATION IN THE LIVER DUE TO 2 : 4–DINITROPHENOL.

A. FONNESU AND CLARA SEVERI.

From the Institute of General Pathology, University of Milan, Italy.

Received for publication October 1, 1954.

SINCE Loomis and Lipmann (1948) discovered that 2 : 4-dinitrophenol (DNP) uncouples oxidation and phosphorylation many investigations have been performed *in vitro* to find the precise mechanism of this effect. However the cellular changes induced by a prolonged administration of DNP *in vivo* have not been examined recently. Older work concerning the toxic properties of this drug and of related compounds has been fully reviewed by several authors (Magne, Mayer and Plantefol, 1932 ; Tainter and Cutting, 1933 ; Heymans and Casier, 1935 ; Staub, 1935).

Recently there has been put forward the hypothesis that the uncoupling of oxidation and phosphorylation could play an important rôle in the pathogenesis of the cellular metamorphoses (Ciaranfi, 1953), and at least as regards the cloudy swelling, such a hypothesis is supported by several experimental results (Fonnesu, 1952, 1954 ; Fonnesu and Severi, 1953, 1954*a*). Thus, experiments have been undertaken to ascertain whether a prolonged DNP-intoxication produced in cells of living animals a morphological alteration, which might be fitted into any of the pictures of classical cell pathology. It has been observed, in the early stages of the intoxication, that both liver and kidney were affected by cloudy swelling while, on the other hand, the other organs examined revealed rather moderate and inconstant lesions. Prolonged treatment with DNP produced in the kidney, in addition to cloudy swelling, the appearance of necrotic foci, while in the liver a quite unexpected change occurred resembling that seen in glycogen storage disease (Fonnesu and Severi, 1954*b*). This last observation appeared of notable interest and deserved further and more extended investigation.

It has recently been observed that liver glycogen is increased in rats poisoned with dinitro-*o*-cresol (DNOC) (Parker, Barnes and Denz, 1951). Immediately after the injection of the drug, liver glycogen decreases, but after a time it tends to increase above normal (Barnes, 1953). According to Barnes there are three possible mechanisms to explain the glycogen accumulation by DNOC : (*a*) delayed absorption of food from the intestine, with the imposition of what amounts to a fast on the animal ; (*b*) action of DNOC on the adrenals, and (*c*) conversion into glycogen by the liver of metabolites which could accumulate during the acute phase of poisoning. The first mechanism, however, appeared improbable, since in fasted rats to which glucose was given orally followed by DNOC the same amount of glycogen was synthesised as in controls receiving glucose alone. Evidence which could decide in favour of one of the other proposed mechanisms has not been obtained. In the experiments with DNOC it has proved impossible to ascertain whether the adrenals play a rôle in bringing about the changes in liver glycogen

levels after DNOC. On the other hand, no experimental result has been found supporting the third hypothesis.

The present paper is concerned with a study, both histological and biochemical, on the accumulation of glycogen in the liver following administration of DNP.

EXPERIMENTAL.

Experimental animals.

Adult Wistar rats (strain WAG-2A) of either sex were used throughout these experiments. The rats were given subcutaneously, twice a day, approximately 10 hr. apart, a saturated aqueous solution of DNP. Each injection was equivalent to 22 mg. DNP/kg. body wt. and the treatment was prolonged for 20 days. Since, at the beginning, the animals were more sensitive to DNP, a slightly lower dose was administered during the first three days (15 mg. DNP/kg. body wt. for each injection). The DNP saturated solution (0·56 per cent) was prepared by dissolving the compound in dilute NaOH, after which the solution was adjusted to pH 7 with dilute HCl. Normal rats of the same stock were used as controls. Care was taken to use for each treated animal a control of the same sex corresponding, as far as possible, in age and weight.

The treated animals were used 15 hr. after the last DNP injection. At this time the rats were less lively than the normal ones on account of the chronic poisoning, but they did not show any signs which could be accounted for by an acute effect of DNP.

Both normal and experimental rats were given *ad libitum* a mixed diet up to 12 hr. prior to the experiment, at which time food was withdrawn. Non-fasting rats were only used when liver glycogen was extracted for the determination of iodine absorption spectra.

The rats were stunned by a blow on the back of the head and then decapitated; pieces of liver, kidney and skeletal muscle were removed as quickly as possible for histological examination and biochemical investigations. In some cases glycogen determinations were also carried out in liver samples kept at room temperature or at 37° for 24 hr. after death.

Experiments (In Vitro).

Enzyme studies in liver.—As soon as removed, the hepatic tissue was homogenised in 2 vol. of ice-cold water. Homogenates were then diluted according to the enzymic activity which had to be assayed. The activities of acid and alkaline phosphatases, of glucose-6-phosphatase and of phosphoglucomutase were measured by the procedure of Cori and Cori (1952).

The reaction systems were incubated in a Warburg apparatus for 1 hr. at 30°. After this period, 1 vol. of 10 per cent trichloroacetic acid was added and inorganic phosphorus was determined in 1 ml. of filtrate. Each experiment included, in addition to the complete systems, samples incubated with water instead of the substrate and others containing the substrate but water in place of the homogenate.

The composition per ml. of the complete reaction mixtures was as follows:

Acid phosphatase: 0·3 ml. of 0·1 M acetate buffer, pH 5; 0·5 ml. of 0·03 M sodium β-glycerophosphate (British Drug Houses Ltd., U.K.), pH 5; 0·2 ml. of homogenate (equivalent to 4 mg. of fresh liver).

Alkaline phosphatase: 0·3 ml. of 0·1 M veronal buffer, pH 8·6; 0·5. ml. of 0·03 M sodium β-glycerophosphate, pH 8·6; 0·2 ml. of homogenate (equivalent to 20 mg. of fresh liver).

Glucose-6-phosphatase: 0·3 ml. of 0·1 M citrate buffer, pH 6·8; 0·5 ml. of 0·01 M potassium glucose-6-phosphate (prepared from the barium salt obtained from Schwarz Laboratories, Inc., U.S.A.), pH 6·8; 0·2 ml. of homogenate (equivalent to 2 mg. of fresh liver).

Phosphoglucomutase: The composition of the reaction mixture was the same as that for glucose-6-phosphatase, but potassium glucose-1-phosphate (Schwarz Laboratories, Inc., U.S.A.) was substituted for potassium glucose-6-phosphate and the substrate solution also contained 0·003 M cysteine (Eastman Kodak Co., U.S.A.) and 0·01 M-$MgCl_2$ to ensure the maximal activity of the mutase. In these experiments easily hydrolysable phosphorus was determined, before and after incubation, in addition to the inorganic phosphorus. As glucose-1-phosphate is totally hydrolysed in 7 min. in 1 N-HCl at 100° and as glucose-6-phosphate is very resistant to acid hydrolysis, the values obtained enabled us to calculate the amount of glucose-1-phosphate converted to glucose-6-phosphate, and how much of the latter remained unchanged and how much was hydrolysed to glucose and inorganic phosphorus.

Isolation and purification of liver glycogen.—Isolation and purification of liver glycogen were performed following essentially the method of Somogyi (1934) with slight modifications which, though causing a loss of some glycogen, made it possible to obtain highly purified glycogen samples. Material for the extractions consisted of several livers obtained respectively from groups of normal and poisoned rats.

Tissues were minced and then digested with 30 per cent NaOH (2 vol. per wt. of tissue) in a boiling water bath for 3 hr. On cooling a layer of soap appeared on the surface of the liquid, from which it was possible to separate the underlying clear fluid. The soapy layer was discarded as proposed by Illingworth, Larner and Cori (1952). From the clear alkaline fluid glycogen was precipitated by adding ethanol until a final alcohol concentration of 33 per cent was reached. After several hours at room temperature, the precipitate was separated by centrifugation, washed repeatedly with a mixture of 1 vol. of ethanol and 2 of 20 per cent NaOH (until the washings appeared colourless) and finally re-washed with ethanol. This impure glycogen was re-dissolved in distilled water and the solution adjusted to pH 4–5 with HCl. Ethanol was added up to a final alcohol concn. of 33 per cent (at which, in an acid medium, glycogen does not precipitate); a brownish precipitate appeared in a few hours, which was separated by centrifugation and discarded. The ethanol concn. in the clear supernatant liquid was then brought to 45 per cent to precipitate the glycogen. This was separated by centrifugation and repeatedly washed with 45 per cent ethanol followed by 95 per cent ethanol and by ether. The ether was allowed to evaporate and the glycogen dried *in vacuo* to constant weight. The purity of the compound was checked by determining the glucose equivalents of a glycogen solution before and after hydrolysis in N-HCl for 4 hr. at 100°. The physical characteristics of isolated glycogen samples were those as described by Somogyi (1934).

Iodine absorption spectrum of liver glycogen.—The iodine absorption spectra of the isolated liver glycogens were determined according to Illingworth and Cori (1952), using a Beckman spectrophotometer mod. DU.

Histological methods.—Sections were stained with haematoxylin and eosin

and with Best's ammoniacal carmine for glycogen. Other sections were stained with iodine solution, before and after incubation with saliva.

Analytical methods.—Glycogen was determined by a modification (Stadie, Haugaard and Marsh, 1951) of the method of Good, Kramer and Somogyi (1933), using the method of Nelson (1944) for the final determination of glucose equivalents.

Phosphorus was determined according to Fiske and SubbaRow (1925).

A Coleman spectrophotometer mod. S.14 was used.

Experiments In Vivo.

Preliminary experiments consisted of blood glucose determinations both in normal and poisoned animals kept without food for 12 hr. Experiments were then performed to investigate the effects of several glycogenolytic agents on the stored glycogen and to study the glucose tolerance. In these experiments the rats had access to food up to 2 hr. prior to the tests. Blood samples were taken by heart puncture before and at various intervals after the injection.

Blood glucose was determined in duplicate according to Nelson's photometric adaptation of Somogyi's method (Nelson, 1944).

Epinephrine.—Epinephrine hydrochloride 1/1000 was injected subcutaneously in doses of 0·08 mg./100 g. body wt.

Hyperglycaemic-glycogenolytic-factor (HGF).—HGF (Eli Lilly and Co., U.S.A., batch No. 208–108B–148A) was used, 2 mg. being dissolved in 2 ml. 0·08 N-NaOH ; the solution was incubated at 39° for 2 hr. and the pH adjusted to 7·4 with dil. HCl. The solution was then diluted to a final vol. of 10 ml. with distilled water and was injected intracardially in doses equivalent to 10 μg. of " crude " HGF/100 g. body wt.

Anterior pituitary somatotropic hormone (STH).—A purified preparation of STH (" Antuitrin Growth," Parke, Davis & Co.) was used. The hyperglycaemic activity of the STH solution was tested in preliminary experiments, since the activity of this preparation decreases progressively with time. Both normal and treated rats received intraperitoneally the smallest dose capable of causing a rapid and transient hyperglycaemia.

Glucose.—The glucose tolerance was determined after intracardiac injection of a 40 per cent glucose solution in doses of 100 mg./100 g. body wt.

Statistical analysis.—All the data were analysed statistically. Symbols and formulae are those given by Fisher (1950).

RESULTS.

Histological Findings.

Liver sections stained with haematoxylin and eosin showed that prolonged poisoning with DNP produced a characteristic change. At low magnifications, trabeculae appeared enlarged and close to one another ; the lobular pattern of the liver was lost. At high magnification, parenchymal cells, with well-defined cell membranes, appeared blown up and tightly packed together. Nuclei were enlarged and slightly paler than normal and the cytoplasm appeared empty (Fig. 2). The degree of change, however, varied from one area to another of the same section.

The histological changes induced by DNP in kidney and skeletal muscle were those observed previously (Fonnesu and Severi, 1954*b*),

Sections stained with Best's ammoniacal carmine showed that normal liver cells contained only a few granules of glycogen, as one would expect, since the livers were obtained from fasting animals (Fig. 3). In contrast, liver cells of DNP-poisoned rats appeared filled with glycogen, although these rats had also fasted for 12 hr. prior to the experiments (Fig. 4). The appearance of sections stained with iodine solution, before and after incubation with saliva, confirmed these results.

Sections of other organs from poisoned animals showed no difference from the normal when stained for glycogen.

Biochemical Investigations.

In Table I the results of glycogen determinations in the tissues of the control and DNP-treated rats are shown. The data are referred to wet wt., since the water content of DNP livers has been found unchanged from the normal (Fonnesu and Severi, 1954*b*).

The glycogen content of the livers from DNP-treated animals was much higher (about 7-fold) than that of the normal rats. On the other hand, the glycogen content of kidney and skeletal muscle from the treated animals was not significantly changed.

TABLE I.—*Glycogen Content of Tissues of DNP-treated Rats Compared with Controls.*
mg. glycogen/100 mg. fresh tissue. Animals fasted for 12 hours.

	Liver.		Muscle.		Kidney.	
	Controls.	DNP-treated rats.	Controls.	DNP-treated rats.	Controls.	DNP-treated rats.
1	0·25	3·10	0·50	0·45	0·06	0·09
2	0·35	1·28	0·35	0·45	0·09	0·05
3	0·10	1·77	0·50	0·32	0·08	0·05
4	0·70	1·60	0·25	0·30	0·07	0·07
5	0·42	2·45	0·62	0·45	0·05	0·05
6	0·55	0·95	0·45	0·35	0·09	0·08
7	0·45	1·90	0·30	0·45	0·04	0·06
8	0·30	0·95	0·42	0·65	0·07	0·07
9	0·25	2·15	0·50	0·50	0·05	0·07
10	0·25	2·35	0·45	0·65	0·06	0·07
11	0·32	1·92	0·52	0·35	—	—
12	0·25	1·35	0·65	0·55	—	—
13	0·40	1·40	0·60	0·60	—	—
14	0·10	1·15	0·35	—	—	—
15	0·25	2·60	0·30	—	—	—
16	0·30	1·65	0·40	—	—	—
17	0·15	1·75	0·40	—	—	—
18	0·10	4·10	—	—	—	—
19	—	2·40	—	—	—	—
20	—	2·30	—	—	—	—
Means ±	0·30±	1·95±	0·44±	0·46±	0·06±	0·06±
S. E. M.	0·037	0·171	0·028	0·033	0·006	0·005
Difference between the means	1·65		0·02		—	
t	8·70		0·46		—	
P	<0·01*		>0·05†		—	

* Highly significant. † Not significant.

In both normal and pathological livers, glycogen totally disappeared when tissues were kept for 24 hr. at room temperature or at 37°.

The results of the enzymic studies in liver are summarised in Table II.

TABLE II.—*Enzymes in Livers of Normal and DNP-treated Rats.*

Mean values of 8 observations: 100 mg. fresh liver, 60 min., 30°.

Enzyme.	Acid phosphatase (P liberated from β-glycerophosphate at pH 5).		Alkaline phosphatase. (P liberated from β-glycerophosphate at pH 8·6).		Glucose-6-phosphatase. (P liberated from glucose-6-phosphate at pH 6·8).		Phosphoglucomutase. (P of glucose-6-phosphate formed from glucose-1-phosphate at pH 6·8).			
							Remained unchanged.		Hydrolysed to inorg. P.	
Normal and treated rats.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.
Mean (μg.)	619	395	33	8	1680	1720	1773	1780	143	122
S.E.M.	85	56	3	0·8	155	143	167	209	69	34
Difference between the means	224		25		20		7		21	
t	2·39		8·5		0·19		0·03		0·27†	
P	<0·05*		<0·01†		>0·05‡		>0·05‡		>0·05‡	

* Significant. † Highly Significant. ‡ Not significant.

The data presented show that both acid and alkaline phosphatase activities were significantly decreased in liver from DNP-poisoned rats. On the other hand, the enzyme directly involved in glycogen degradation in liver, namely glucose-6-phosphatase, appeared unchanged. In the experiments with glucose-1-phosphate as substrate, in which maximal activity of phosphoglucomutase was ensured by the addition of cysteine and $MgCl_2$, the same amount of glucose-1-phosphate was converted into glucose-6-phosphate by both normal and pathological livers. The quantity of the latter hydrolysed to glucose and inorganic phosphorus was the same when the systems contained either normal or pathological liver homogenates. Consequently no difference appeared in the amount of glucose-6-phosphate, which was found unchanged at the end of incubation.

The iodine absorption spectra of the glycogen isolated from livers of normal and poisoned rats were practically identical and appeared equally chromogenic with an absorption peak at 470 mμ. The data concerning the glycogen of normal rat liver correspond to those found by Illingworth and Cori (1952) for glycogen of normal human liver.

Table III states the blood glucose level in normal and DNP-treated rats fasted for 12 hr. In DNP rats the level is significantly higher than in the controls.

The results concerning the effects of the glycogenolytic agents and the glucose load are presented in the form of curves showing average changes in blood glucose concentration. It will be observed that the blood glucose remained practically constant during the control period preceding the injections.

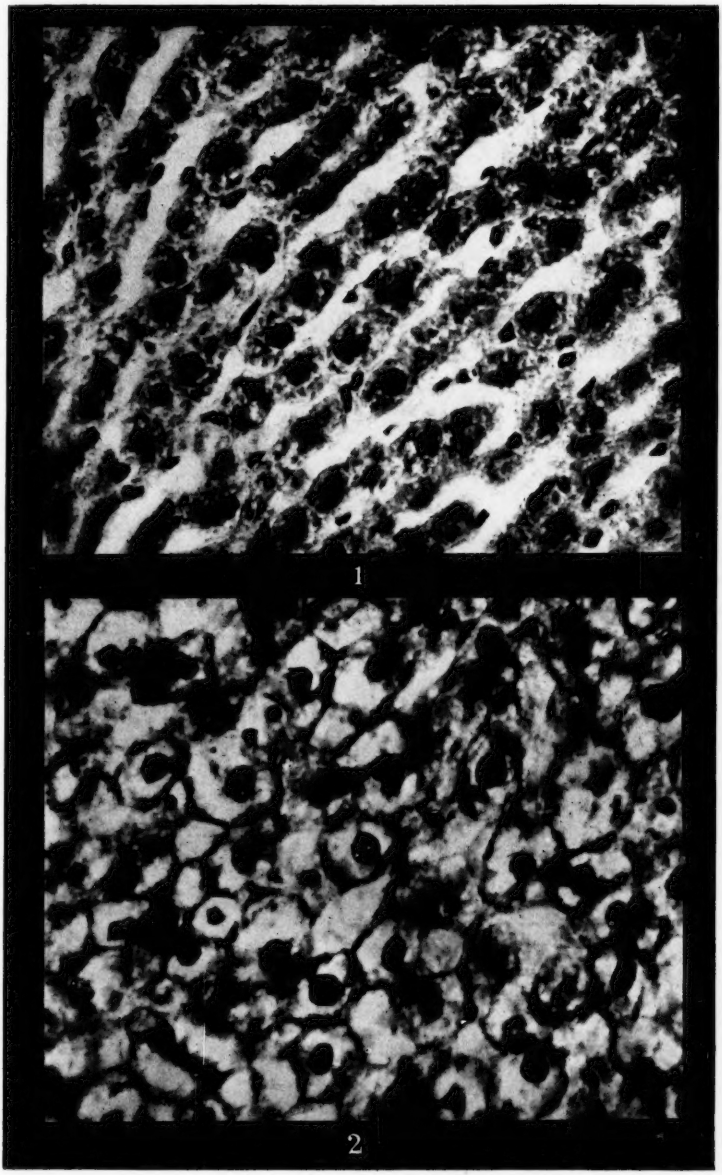
EXPLANATION OF PLATES.

FIG. 1.—Liver of normal rat fasted for 12 hours. Haematoxylin and eosin. (× 560).

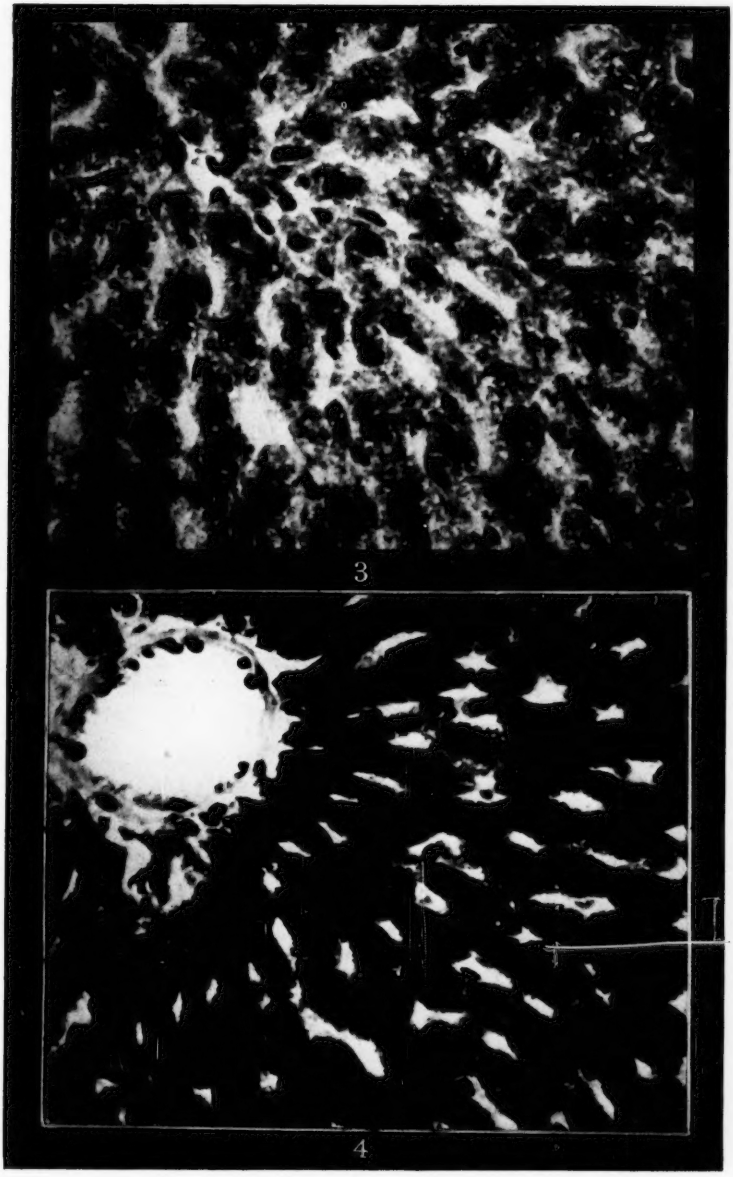
FIG. 2.—Liver of DNP-treated rat fasted for 12 hours. Haematoxylin and eosin. (× 560).

FIG. 3.—Liver of normal rat fasted for 12 hours. Best's ammoniacal carmine for glycogen. (× 560).

FIG. 4.—Liver of DNP-treated rat fasted for 12 hours. Best's ammoniacal carmine for glycogen. (× 560).



Fonnesu and Severi.



3

4

Fonnesu and Severi.

TABLE III.—*Blood Glucose Concentration in Normal and DNP-treated Rats Fasted for 12 Hours (mg./100 ml.).*

	Normal.	DNP-treated.
1	78	90
2	76	114
3	74	100
4	88	108
5	86	100
6	96	102
7	82	108
8	70	112
9	82	104
10	86	132
11	88	103
Means ± S. E. M.	82±2·26	106±3·21
Difference between the means	24	
t	6·1	
P	<0·01*	

* Highly significant.

Fig. 5 represents experiments in which rats received a subcutaneous injection of epinephrine hydrochloride. Following the injection the blood glucose rose in both control and test animals, but the hyperglycaemic response was much greater in the latter. In normal rats the peak rise was reached in about 2 hr. and was followed by a rapid decrease. In the treated rats the glucose rise was much greater, and the maximum was reached after 5 hr.

The experiments in which animals were given HGF intracardially gave results shown in Fig. 6. The injection of HGF was followed, in both normal and DNP-poisoned rats, by a prompt and marked hyperglycaemia, but the increase was more pronounced and prolonged in DNP-treated rats than in controls. Taking into account that large doses of HGF were used to obtain the maximal change, our data for normal animals appear well in agreement with the results of others (Foá, Santamaria, Berger, Smith, and Weinstein, 1952 ; Kirtley, Waife and Peck, 1953 ; Ingle, Nezamis and Humphrey, 1953).

The hyperglycaemic response produced by STH in normal and treated rats (Fig. 7) was similar to, although much smaller than that produced by epinephrine or HGF.

Fig. 8 shows that the glucose tolerance was markedly reduced in DNP-poisoned rats as compared with normal controls.

DISCUSSION.

Evidence has been presented (Fig. 1–4 ; Table I) that in rats treated repeatedly with DNP, a glycogen accumulation occurs in the liver in the later stages of the treatment. Such a change is strictly limited to the liver, since in the other organs the glycogen levels appear unaltered from the normal. It has already been mentioned that analogous results have been obtained by poisoning rats with DNOC (Parker *et al*, 1951 ; Barnes, 1953), a compound similar to DNP. These observations undoubtedly are unexpected because it is known that liver glycogen decreases after a single injection of DNP or DNOC.

The change seen in the liver following prolonged treatment with DNP resembles a picture characteristic in human pathology, known as von Gierke's or glycogen storage disease (for literature see van Creveld, 1939). In von Gierke's disease, however, glycogen storage, which may be enormous, is generally not limited to the liver, but occurs also in other organs such as the kidney, heart, muscles and brain ; large amounts of fat are also present in the tissues. The deposits of both

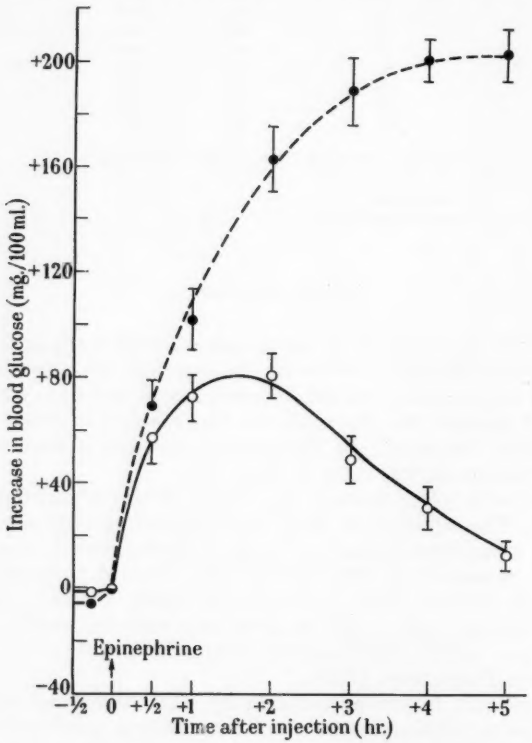


FIG. 5.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - -●) following an epinephrine injection. Each point represents the mean of six animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

glycogen and fat cause a marked enlargement of the organs, with the exception of the spleen, the hepatomegaly without splenomegaly being characteristic for the condition. In patients with von Gierke's disease, the liver glycogen is not readily available for blood glucose formation as shown by the tendency to hypoglycaemia, by the flat blood glucose curve after epinephrine injection and by the persistence of the glycogen in tissues for an unlimited time after death. In contrast, in DNP-poisoned rats the glycogen accumulates only in the liver and disappears totally after death. As compared with normal controls, the DNP

rats exhibit a greater blood glucose concentration (see Table III) and a higher blood sugar curve after epinephrine injection (see Fig. 5). On the other hand, the fat content in DNP livers is lower than normal (Fonnesu and Severi, 1954*b*), this being a further difference from von Gierke's disease.

It should be noted here that the levels of liver glycogen found in fasted DNP animals (Table I) can occur in well fed normal rats, though they are not found in

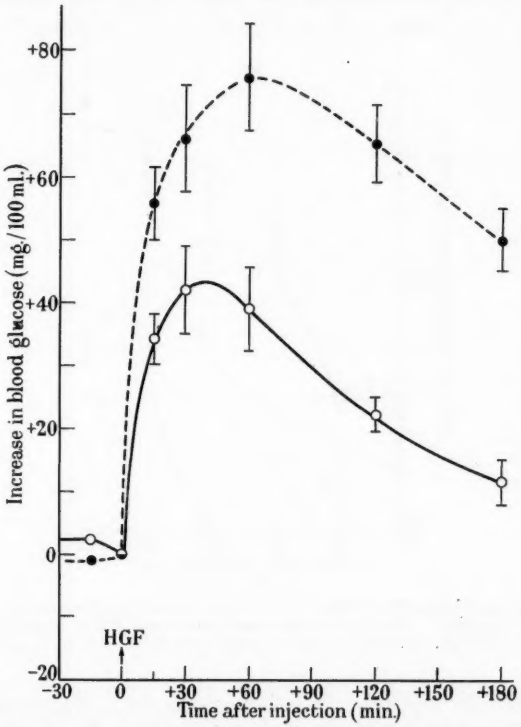


FIG. 6.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - -●) following a HGF injection. Each point represents the mean of six animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

normal fasted rats. Therefore, the enlargement of the cells in DNP livers (Fig. 2) cannot be attributed exclusively to the glycogen accumulation. Since cloudy swelling occurs in liver in the early stages of DNP-poisoning (Fonnesu and Severi, 1954*b*), it is reasonable to assume that glycogen accumulates in swollen cells. This view is supported by the finding that nuclei are also markedly enlarged (Fig. 2) as compared with the normal (Fig. 1).

The following considerations led us to investigate the glucose-6-phosphatase activity in livers from normal and DNP-poisoned animals. The conversion of glycogen to glucose in the liver requires several enzymes, *viz.*, phosphorylase, amylo-1 : 6-glucosidase (Cori and Larner, 1950), phosphoglucomutase and glucose-

6-phosphatase. For glycogen synthesis from glucose hexokinase and the branching enzyme are required in addition to phosphorylase and phosphoglucomutase. Thus two enzymes, amylo-1 : 6-glucosidase and glucose-6-phosphatase, bring about glycogen degradation without intervening in its synthesis. A reduction of these enzymes could cause glycogen storage in the liver, since a deficiency of enzymes also involved in glycogen synthesis would have been incompatible with the accumulation of the polysaccharide in liver. However, the post-mortem glycogen disappearance

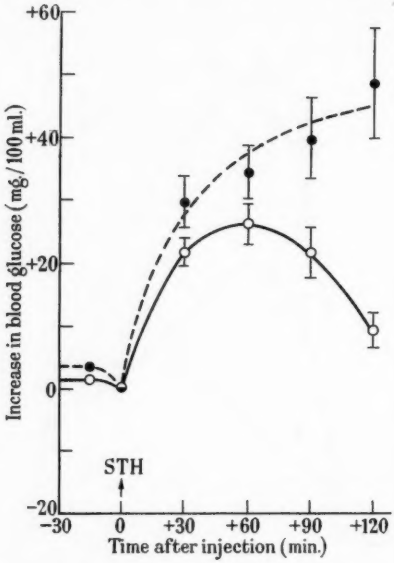


FIG. 7.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - -●) following a STH injection. Each point represents the mean of nine animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

evidently excluded, in our case, a defect of amylo-1 : 6-glucosidase, by whose action the complete splitting of the glycogen molecule by phosphorylase is conditioned (Cori and Larner, 1951). Since the glucose-6-phosphatase activity has been measured and found unchanged (Table II), it is possible to conclude that the glycogen liver storage by DNP does not depend on a deficiency of the enzymes involved in glycogen degradation. As can be seen from the data summarised in Table II, the decrease of acid and alkaline phosphatases is the only enzymic change observed in DNP livers. This enzymic deficiency, however, cannot account for the glycogen accumulation in liver and must be regarded as a concomitant disturbance.

The glycogen accumulation in DNP treatment could not be attributed to an abnormality in the structure of glycogen such that the enzymes were unable to attack the polysaccharide molecule. In fact, apart from the post-mortem glycogen disappearance in DNP livers, there is evidence that carbohydrate stores in poisoned animals are well mobilised by glycogenolytic agents (see Fig. 5–7). However,

this does not exclude the possibility that some structural alterations, compatible with the enzymic degradation, might exist in the glycogen molecule. Therefore it appeared of interest to ascertain whether or not the liver glycogen formed after DNP poisoning exhibited a normal iodine absorption spectrum. It is known that the absorption spectrum of polysaccharides in the presence of iodine may give information about the structure of the polysaccharide. However, the iodine absorption spectrum of liver glycogen from poisoned animals is normal.

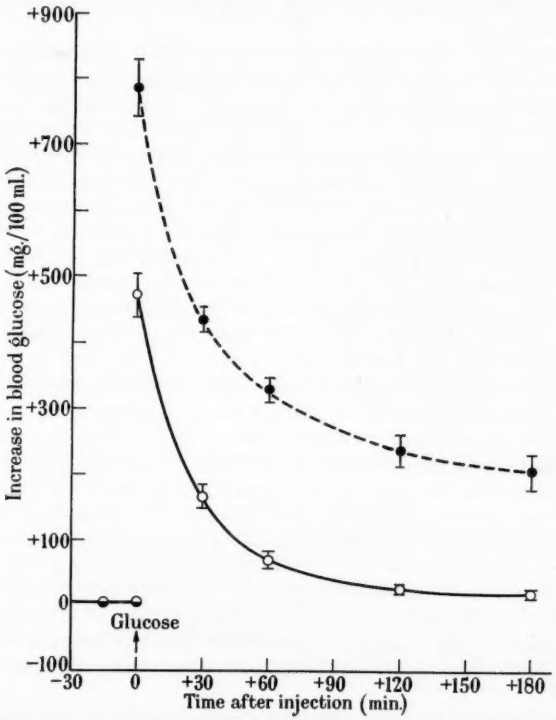


FIG. 8.—Glucose tolerance curve in normal (○———○) and DNP-treated rats (●- - - - -●). Each point represents the mean of eight animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

The changes in blood glucose induced by epinephrine, HGF and STH, both in normal and intoxicated rats, merit some discussion. It has been long known that epinephrine and HGF produce hyperglycaemia in normal animals by causing increased hepatic glycogenolysis. Sutherland and Cori (1951) have shown that they increase the concentration of active phosphorylase in liver, this effect being sufficient to explain their hyperglycaemic action. According to these authors, the phosphorylase content of the liver would not be static, but would represent a balance between an active and an inactive form of the enzyme. The rapid change in either direction would be brought about by an enzyme system in the liver upon which epinephrine and HGF would act.

It appears evident that the blood glucose rise after the injection of glycogenolytic agents depends on an adequate supply of liver glycogen. When the glycogen stores are depleted as in fasting (Weisberg, Caren, Huddlestun and Levine, 1949 ; Pincus, 1950) and uncontrolled experimental diabetes (Pincus, 1950), the effects of glycogenolytic agents do not fully manifest themselves.

These considerations led us to investigate the effects of epinephrine and HGF on the liver glycogen of poisoned rats. Both epinephrine and HGF produced in DNP-treated animals a greater and more prolonged hyperglycaemia than in controls (Fig. 5 and 6).

One might suppose that the glycogen liver accumulation was due to a deficiency of HGF. Experiments with HGF did not prove this, since they showed only that the HGF injected was active on liver glycogen. However, the above supposition might be easily controlled by investigating the effects of a factor stimulating HGF secretion. For this purpose STH was used because this hormone, in addition to reducing insulin secretion (Anderson and Long, 1947) and inhibiting peripheral utilisation of glucose (Milman, DeMoor and Lukens, 1951), produces hyperglycaemia by stimulating the alpha cells of the pancreas to secrete HGF (Foá, Magid, Glassman and Weinstein, 1953). A comparison of Fig. 6 and 7 shows that the blood glucose changes after STH were very similar to those after HGF. The smaller hyperglycaemic response after STH can be explained by the fact that the amount of HGF secreted under STH stimulation was certainly much smaller than that of HGF injected directly.

The results obtained with epinephrine, HGF and STH concordantly show that in rats treated with DNP the blood glucose rise is more pronounced than in normal controls after the injection of glycogenolytic agents. This fact certainly depends on the increased glycogen content of the liver of intoxicated rats. It seemed possible, however, that at least in part it may be due to a reduced glucose utilisation by the tissues because of biochemical alterations induced by DNP.

It appeared, therefore, of interest to investigate the glucose tolerance, which was seen to be markedly reduced in DNP-treated animals (Fig. 4). The decreased rate of disappearance of the injected glucose from the blood cannot be attributed to the raising of renal threshold. It has been observed that the threshold for glucose is lowered in the kidney in cloudy swelling (Agostini, 1953) and that the kidneys of DNP-treated rats show parenchymatous degeneration (Fonnesu and Severi, 1954*b*).

It seems reasonable to conclude that the reduced glucose tolerance is due to a decreased utilisation of the injected glucose. This conclusion is also supported by the higher blood glucose level in fasting DNP-treated rats.

With this in mind, it is possible to attempt the interpretation of the glycogen accumulation by DNP. The reduced glucose utilisation in peripheral tissues caused by DNP would lead to the raising of blood glucose level, which would induce the glycogen accumulation in liver through the known homoiostatic mechanism (Soskin and Levine, 1946 ; Frank, 1949). The equilibrium between formed glycogen and split glycogen would thus be altered in favour of the former. It is evident, however, that in this case it is necessary to concede that the DNP liver must have a normal or only slightly decreased capacity of synthesising glycogen. Thus, the alteration leading to glycogen accumulation will not lie in the liver but in the decreased glucose utilisation in the peripheral tissues which will produce hyperglycaemia.

The proposed mechanism recalls the effects of the adrenocortical hormones in the carbohydrate metabolism, *viz.*, (*a*) increased gluconeogenesis, (*b*) marked increase of liver glycogen with slight modification of the glycogen muscle content, and (*c*) reduced peripheral utilisation of carbohydrates. Whether the results obtained are attributable to a direct action by DNP or to an indirect action mediated by the adrenals remains to be established.

SUMMARY.

It has been shown by histological and chemical methods that on prolonged treatment of rats with DNP a glycogen accumulation takes place in the liver.

Liver glycogen disappeared totally after the death of the animal.

The blood glucose level in fasting DNP-treated rats was raised.

Glucose-6-phosphatase and phosphoglucomutase were unchanged in the liver of DNP-poisoned rats, whilst both acid and alkaline phosphatases were decreased.

The iodine absorption spectrum of glycogen isolated from livers of DNP rats was unmodified as compared with the spectrum of glycogen from normal rat liver.

The effects of epinephrine and HGF on liver glycogen have been investigated. Both these glycogenolytic agents produced in DNP-treated animals a greater and more prolonged hyperglycaemia than in normal controls. Analogous results were obtained by stimulating the secretion of HGF with STH.

The glucose tolerance was markedly reduced in DNP-treated rats.

A hypothesis explaining the action of DNP in causing glycogen accumulation is proposed.

This investigation has been supported by a grant from the " Lega Italiana per la Lotta contro i Tumori".

While this paper was in preparation, a paper by Smith (1954) was published showing that the effect of sodium salicylate on liver glycogen and blood glucose of rats was similar to that of DNP. A state of eucorticalism, rather than a stimulation of the adrenal cortex, is sufficient for the effect of salicylate on liver glycogen and blood glucose to be demonstrated. It is probable that the mechanisms involved in the salicylate effect are the same as those of DNP and DNOC.

REFERENCES.

Agostini, C.—(1953) *Boll. Soc. ital. Biol. sper.*, **29**, 278.
Anderson, E. and Long, J. A.—(1947) *Endocrinology*, **40**, 98.
Barnes, J. M.—(1953) *Biochem. J.*, **54**, 148.
Ciaranfi, E.—(1953) *Atti Soc. ital. Pat.*, **3**, 7.
Cori, G. T. and Cori, C. F.—(1952) *J. biol. Chem.*, **199**, 661.
Idem and Larner, J.—(1950) *Fed. Proc.*, **9**, 163.—(1951) *J. biol. Chem.*, **188**, 17.
Creveld, S. van—(1939) *Medicine*, **18**, 1.
Fisher, R. A.—(1950) 'Statistical Methods for Research Workers.' Edinburgh (Oliver & Boyd).
Fiske, C. H. and Subbarow, Y.—(1925) *J. biol. Chem.*, **66**, 375.
Foá, P. P., Magid, E. B., Glassman, M. D. and Weinstein, H. R.—(1953) *Proc. Soc. exp. Biol., N.Y.*, **83**, 758.
Idem, Santamaria, L., Berger, S., Smith, J. A. and Weinstein, H. R.—(1952) *Ibid.*, **80**, 635.

FONNESU, A.—(1952) *Boll. Soc. ital. Biol. sper.*, **28**, 482.—(1954) *Arch. Sci. biol.*, **38**, 1.
Idem AND SEVERI, C.—(1953) *G. Biochimica*, **2**, 326.—(1954*a*) *Experientia*, **10**, 28.—(1954*b*) *Arch. Sci. biol.*, **38**, 597.
FRANK, E.—(1949) 'Pathologie des Kohlenhydratstoffwechsels.' Basel (Benno Schwabe & Co.).
GOOD, C. A., KRAMER, H. AND SOMOGYI, M.—(1933) *J. biol. Chem.*, **100**, 485.
HEYMANS, C. AND CASIER, H.—(1935) *Arch. int. Pharmacodyn.*, **50**, 20.
ILLINGWORTH, B. AND CORI, G. T.—(1952) *J. biol. Chem.*, **199**, 653.
Idem, LARNER, J. AND CORI, G. T.—(1952) *Ibid.*, **199**, 631.
INGLE, D. J., NEZAMIS, J. E. AND HUMPHREY, L. M.—(1953) *Proc. Soc. exp. Biol., N.Y.*, **84**, 232.
KIRTLEY, W. R., WAIFE, S. O. AND PECK, F. B.—(1953) *Ibid.*, **83**, 387.
LOOMIS, W. F. AND LIPMANN, F.—(1948) *J. biol. Chem.*, **173**, 807.
MAGNE, H., MAYER, A. AND PLANTEFOL, L.—(1932) *Ann. Physiol. Physicochim. biol.*, **8**, 1.
MILMAN, A. E., DEMOOR, P. AND LUKENS, F. D. W.—(1951) *Amer. J. Physiol.*, **166**, 354.
NELSON, N.—(1944) *J. biol. Chem.*, **153**, 375.
PARKER, V. H., BARNES, J. M. AND DENZ, F. A.—(1951) *Brit. J. industr. Med.*, **8**, 226.
PINCUS, I. J.—(1950) *J. clin. Endocrin.*, **10**, 556.
SMITH, M. J. H.—(1954) *Biochem. J.*, **57**, 349.
SOMOGYI, M.—(1934) *J. biol. Chem.*, **104**, 245.
SOSKIN, S. AND LEVINE, R.—(1946) 'Carbohydrate Metabolism.' Chicago (The University of Chicago Press).
STADIE, W. C., HAUGAARD, N. AND MARSH, J. B.—(1951) *J. biol. Chem.*, **188**, 167.
STAUB, H.—(1935) *Klin. Wschr.*, **14**, 185.
SUTHERLAND, E. W. AND CORI, C. F.—(1951) *J. biol. Chem.*, **188**, 531.
TAINTER, M. L. AND CUTTING, W. C.—(1933) *J. Pharmacol.*, **49**, 187.
WEISBERG, H. F., CAREN, R., HUDDLESTUN, B. AND LEVINE, R.—(1949) *Amer. J. Physiol.*, **159**, 98.

THE CONSTITUTION OF HUMAN RENAL RETICULIN

G. M. WINDRUM, P. W. KENT AND J. E. EASTOE.

From the Department of Pathology, Radcliffe Infirmary, Oxford, the Department of Biochemistry, University of Oxford, and the British Gelatine and Glue Research Association, London.

Received for publication September 29, 1954.

RETICULIN fibres were first described by Kupffer (1876), and as long ago as 1888, Mall claimed that a chemical difference existed between these reticulin fibres and collagen, since only the latter produced gelatin on boiling with water. The demonstration by Maresch (1905) that an ammoniacal silver nitrate solution (Bielschowasky, 1905) used for the histological impregnation of neurofibrils, would also demonstrate reticulin, but not collagen fibres, seemed to be additional evidence for a difference between the two substances. This difference was challenged by Mallory and Parker (1927), who pointed out that relatively minor differences in staining reactions may not represent significant differences in chemical structure. Noting the close histological relations of reticulin and collagen, the general similarity in staining reactions, and the apparent conversion of reticulin to collagen in sclerosing processes, they asserted : " Reticulin as a chemically distinct intercellular substance does not exist ; it is collagen in a separated form, rendered prominent by the silver stain ".

Foot (1928) did not accept this view, and attempted to prove, by a combined histological and biochemical approach, that there were significant differences between these tissue elements. He concluded that reticulin and collagen were closely related but distinct substances, and suggested that reticulin may be a precursor of collagen.

Macfarlane and MacLennan (1945) reported that toxic filtrates of *Clostridium welchii* contained a distinct enzyme capable of hydrolysing collagen, and Robb-Smith (1945) was able to demonstrate histologically that this enzyme attacked reticulin and collagen equally rapidly. With the introduction of the periodic acid-Schiff (P.A.S.) reaction to histochemistry (McManus, 1946 ; Hotchkiss, 1948) it was noted that reticulin fibres gave a positive reaction (Lillie, 1947 ; Robb-Smith, 1952), while collagen gave only a faint colouration. Robb-Smith (1952) considered this latter as a negative reaction. Lillie (1953), however, insisted that collagen also gave a positive P.A.S. reaction. With the sulphation-metachromasia reaction (Kramer and Windrum, 1953 ; 1954), the two types of fibres give quite different results, the reticulin staining a red metachromatic colour, and collagen a blue orthochromatic colour. The results obtained with these histochemical tests suggest the possible presence in reticulin of appreciable carbohydrate residues not present in collagen.

Bowes and Kenten (1949) compared the amino acid composition of reticulin and collagen by paper chromatography. In reticulin from the lymph nodes and adipose tissue of the ox a general similarity of amino acid composition was found, though reticulin apparently had lower proline and hydroxyproline values than collagen.

The present paper describes the isolation of a sample of histologically defined reticulin, its biochemical analysis and some conclusions as to the relationship of reticulin to collagen.

Table I summarises the more important histological criteria that may be used to differentiate collagen and reticulin fibres.

TABLE I.—*Staining Properties of Collagen and Reticulin.*

	Collagen.	Reticulin.
Pattern	Bundles of non-branching fibres	Single branching fibres
Silver impregnation	Yellow-brown	Black
Acid fuchsin	Bright red	Faint red
Periodic acid-Schiff	Faint pink	Bright red
Sulphation-metachromasia	Blue	Red

MATERIALS AND METHODS.

Isolation of Reticulin.

The biggest problem is to find a source of histologically acceptable reticulin unassociated with collagen. Kramer and Little (1953) drew attention to the fact that in the normal adolescent kidney, the connective tissue stroma of the cortex consists almost entirely of reticulin. This view was first expressed by Mall (1896), who regarded the intertubular stroma of the renal cortex, including the basement membrane of the tubules and Bowman's capsule, as all purely reticulin.

The method used to separate reticulin from other tissue components was as follows :

Kidneys were selected from subjects aged 15–25 years, dying of a cause unrelated to renal disease. Selected areas of these kidneys were prepared for paraffin sections to confirm the absence of any unsuspected renal lesion. The remainder of the kidney tissue was washed briefly in 0·85 per cent NaCl, and then frozen in the freezing compartment of a refrigerator to facilitate the next step.

This consisted of separating the midcortical zone from the rest of the kidney. Slices 0·5–1 mm. thick were cut with a sharp knife, and the outer and inner zones of the cortex removed by careful dissection to remove capsule and blood vessels, which contain associated collagen. The remaining midcortical area consists of a large number of glomeruli and adjacent tubules, together with small blood vessels, whose walls may contain slight amounts of elastin and collagen, but this admixture with the reticulin is negligible.

These thin tissue slices were placed in jars containing 0·05 N–NaOH to remove the cellular materials, a small crystal of thymol being added to inhibit bacterial growth. The extracting fluid was changed at 3-hr. intervals for 12 hr. and the material left in it overnight, the process being repeated on the following day ; then 0·85 per cent NaCl was added to dilute the remaining NaOH that was not easily decantable. After 12 hr., the material was continuously washed in 0·85 per cent NaCl for 48 hr. The remaining solid material was washed in distilled water, then in chloroform-methanol (equal volumes) for 24 hr., acetone for 24 hr., and dried in an incubator at 37°.

During extraction, small pieces of tissue were examined histochemically. After the saline washing, these showed the exclusive presence of cell-free fibrillary material having a positive P.A.S. reaction, and becoming metachromatic after sulphation.

Chemical Investigations and Results.

Prior to the chemical analyses, samples were ashed and the moisture content was assessed so that results could be expressed on an ash-free, moisture-free basis.

A qualitative test with the anthrone reagent produced a positive reaction with the intact reticulin, showing the presence of carbohydrate. In preliminary experiments, the material was hydrolysed in 5 N–HCl in a sealed tube for 6 hr. at 110°. The reticulin dissolved slowly,

producing a yellow-brown solution and a brown residue. Chloroform extraction of the hydrolysate produced a chloroform-soluble fraction, presumably of a lipid nature. The hydrolysate also contained a mixture of amino acids and reducing sugars.

Amino acids.

Identification of the amino acids was attempted by paper chromatography, following the general method of Consden, Gordon and Martin (1944).

Reticulin was hydrolysed with 5 N–HCl in a sealed tube at 110° for 6 hr. The HCl was removed over NaOH *in vacuo*, and part of the residue taken up in distilled water and analysed chromatographically. The amino acids were revealed by spraying the paper with ninhydrin reagent, the colouration being produced after heating the chromatogram paper at 110° for 10 min.

Using a number of chromatograms and test amino acids, it was possible to show the probable presence of alanine, glycine, valine, leucine, hydroxyproline, proline, phenylalanine, serine, threonine, arginine, aspartic acid and glutamic acid. All these amino acids are present in collagen, and it seemed that the provisional identification of the amino acids by this method would give a pattern similar to that of collagen. Cystine and tyrosine appeared to be absent on the chromatograms of the reticulin hydrolysates.

A complete amino acid analysis of the reticulin was carried out using the method of Moore and Stein (1951). After hydrolysis for 24 hr. in 6 N–HCl at 100°, the acidic and neutral amino acids were separated on a 100 × 0·9 cm. column of Dowex 50 resin (200–400 mesh, 12 per cent cross linking) and the basic amino acids on a 15 × 0·9 cm. column. One ml. fractions of the eluant were collected using a specially designed drop counting fraction collector. The fractions were brought to pH 5·0 by addition of suitable small volumes of standard acid or alkali (Eastoe and Eastoe, 1954) and heated at 100° for 20 min. with 2 ml. ninhydrin reagent (Moore and Stein, 1948). The optical density of the blue solutions thus formed was measured using a Unispek spectrophotometer at 670 mμ. The yellow colour due to proline was measured at 440 mμ. Hydroxyproline was determined independently by the method of Neuman and Logan (1950) on the unfractionated hydrolysate.

The amino acid peaks were identified by their order of emergence from the column, and their relative positions corresponded very closely with those stated by Moore and Stein (1951). The general appearance of the chromatogram corresponded with those of mammalian collagen and gelatin. A trace of ninhydrin-positive material having negligible retention volume and emerging at fraction 23 was assumed to be cysteic acid, probably formed from cysteine during the acid hydrolysis. A small peak emerging just ahead of hydroxyproline was identified as methionine sulphoxide (Schram, Dustin, Moore and Bigwood, 1953). This was undoubtedly formed from methionine by oxidation during the preliminary hydrolysis. A small peak was found in the position usually occupied by glucosamine during the elution from the 15 cm. column (Eastoe, 1954).

Table II shows the amino acid composition of reticulin, compared with the reported values for collagen (Bowes , Elliot and Moss, 1953). This shows a close similarity between the composition of collagen and of the protein fraction of reticulin.

Carbohydrate.

Total carbohydrate was estimated by the anthrone reaction (Dreywood, 1946) following the modification of Bangle and Alford (1954). Five samples of reticulin (weighing 8·8, 14·4, 21·5, 25·5 and 26·5 mg.) were each treated with 2 ml. of distilled water, followed by 4 ml. of the anthrone reagent (0·16 per cent anthrone in 96 per cent H_2SO_4). A similar procedure

TABLE II.—*Amino Acid Analyses.*

(Amino acid nitrogen as percentage of total nitrogen.)

	Reticulin.	Collagen.
Alanine	7·68	8·0
Glycine	24·60	26·3
Valine	2·13	2·2
Leucine	2·85	3·2
Isoleucine	1·43	
Proline	7·73	9·9
Phenylalanine	1·44	1·1
Tyrosine	0·24	0·6
Serine	3·40	2·5
Threonine	1·74	1·5
Cysteic acid	0·04	—
Methionine	0·58	0·4
Methionine sulphoxide	0·10	—
Arginine	14·43	15·3
Histidine	1·27	1·2
Lysine	3·44	4·7
Aspartic acid	4·21	3·6
Glutamic acid	6·10	5·8
Amide N_2	3·42	3·5
Hydroxyproline	8·56	7·0
Hydroxylysine	1·93	1.2
Hexosamine	0·07	0·14
Total	97·39	

was followed for a series of galactose standards. After 15 min. the solutions were filtered through glass wool and the colourations measured in a Spekker photometer at 600–680 mμ. Under these conditions, no colouration resulted with a comparable amount of glucosamine. Values of 3·8, 4·3, 4·1, 4·7 and 4·0 per cent were found for the carbohydrate content (calculated as galactose) of the five samples of reticulin respectively.

To determine which carbohydrates were present, descending chromatograms were run in *n*-butanol ; ethanol ; water (40 : 10 : 50). Test substances were added to compare the chromatographic mobilities, and the sugars revealed by spraying with the aniline hydrogen phthalate reagent of Partridge (1949).

Galactose, mannose and fucose were present, as well as a small amount of glucosamine. No glucose appeared to be present.

Hexosamine.

Since the anthrone reagent estimates only non-nitrogenous carbohydrate, the hexosamine content was determined separately. This was performed by a modification of the method of Elson and Morgan (1933). A sample of reticulin was weighed into a tube, 2 ml. of 5 N–HCl were added, and hydrolysis carried out at 110° for 6 hr. after the tube had been sealed. The tube was opened, and the hydrolysate dried *in vacuo* over NaOH. This dried hydrolysate was taken up in 0·5 ml. of 0·3 N–HCl, transferred to a test tube, and 1·0 ml. of acetyl acetone solution added (1·5 ml. acetyl acetone made up to 50·0 ml. with 1·25 N–Na_2CO_3). This was heated for 1 hr. in a water bath at 95° and allowed to cool, when 10 ml. of 95 per cent ethanol and 1·0 ml. of Ehrlich's reagent (1·6 gm. *p*-dimethylaminobenzaldehyde in 30 ml. concentrated H_2SO_4 and 30 ml. ethanol) were added. After standing for 1 hr., the intensity of the red colouration produced was read against a blank (0·5 ml. of 0·3 N–HCl treated as above) and a number of standards prepared from a solution of glucosamine of known strength, in a Spekker photometer.

Two readings were made on reticulin samples weighing 6·7 mg. and 48·7 mg., values of 0·17 per cent and 0·16 per cent hexosamine being obtained.

To determine whether glucosamine and galactosamine were both present, 370 mg. of reticulin were hydrolysed, as described previously, and dried. Separation of the hexosamines was then done on an ion exchange resin column, using the method of Gardel (1953).

A small column (40 cm. × 0·7 cm.) was packed with Dowex 50 resin (200–400 mesh) converted to its acid form with 5 N–HCl and thoroughly washed with 0·3 N-HCl. The hydrolysate of reticulin was dissolved in a little 0·3 N–HCl and placed on top of the column. The resin was eluted with 0·3 N–HCl at the rate of 2 ml. per hour. Separate fractions of eluate (0·5 ml.) were collected mechanically. The fractions were examined for the presence of amino sugar by the above modification of the Elson and Morgan procedure. Under these conditions, glucosamine and galactosamine are successively and quantitatively eluted from the column. With hydrolysed reticulin only a single amino sugar component corresponding to glucosamine was observed.

Uronic acid.

A qualitative test for uronic acid, based on the naphtho-resorcinol method of Tollens (1908), gave a negative result, so it was presumed that the carbohydrate of reticulin was uronic acid-free. This result may be viewed with some caution, as uronic acid decomposes rapidly under acid hydrolysis conditions, and only traces may remain of the original amounts present.

Ester sulphate.

A quantity of the dried hydrolysate of reticulin was taken up in distilled water. A solution of barium chloride added to this failed to produce a precipitate, presumptive evidence for the absence of sulphate groups.

Lipid.

Initially it was thought that the chloroform-soluble fraction obtained from hydrolysis of reticulin represented lipid derived from extrareticular sources that had not been completely removed in the preparation of the samples. To free the reticulin from this contamination, it was extracted with boiling chloroform-methanol (equal volumes) in a Soxhlet extractor for 48 hr. This is said (Pearse, 1953) to be capable of removing all but bound lipids. This procedure resulted in a very small weight loss in some samples, the highest loss recorded being 4 per cent; on repeating the extraction, no further lipid was obtained and the weight of reticulin remained constant. Hydrolysis of this solvent-extracted material in 5 N–HCl in a sealed tube for 6 hr. at 110° yielded a chloroform-soluble fraction as before. It was then concluded that a lipid is, in fact, an integral part of the structure of the reticulin.

Three samples of this solvent-extracted material (weighing 104, 128 and 370 mg.) were taken; to each was added 7 ml. of 5 N–HCl, the tube sealed and heated for 6 hr. at 110°. At the end of this time the tubes were opened, and the hydrolysates repeatedly extracted with chloroform. The combined chloroform extracts were dried with magnesium sulphate and filtered into a weighed flask. The chloroform was then evaporated off under reduced pressure, and the flask and extracted material re-weighed. Values of 11·6, 11·5 and 9·6 per cent lipid were obtained for the three samples respectively.

The Liebermann Burchard reaction for cholesterol was negative. Ultra-violet absorption of the material dissolved in methanol showed only generalised absorption between 245 and 280 mμ, with no specific absorption bands, suggesting the absence of aromatic and conjugated systems.

The material was found to contain no phosphorus, and from all tests it seemed that the material was a simple fatty acid with melting point 49–52°.

A sample of this fat was converted to its methyl ester, and submitted to Drs. Cropper and Heywood (I.C.I., Manchester) for determination of its type by vapour-phase chromatography (Cropper and Heywood, 1953).

Several runs were carried out on a sodium chloride-silicone grease column at 185°, using 0·02 ml. each time. Two bands, which (by comparison with the band due to added methyl laurate) could be identified as being due almost certainly to methyl myristate and methyl palmitate, were present. It was estimated that there was approximately 5 per cent palmitate, with the rest myristate. There was also a suggestion of a tiny band which could be due to methyl octanoate, but the amount was very small ($< 0{\cdot}5$ per cent).

DISCUSSION.

The general behaviour of the reticulin on hydrolysis was different from that of collagen. Collagen will dissolve rapidly and completely on heating with 5 N–hydrochloric acid, whereas the reticulin dissolved very slowly and gave rise to a much larger quantity of a brown residue. This behaviour suggested that reticulin may be a fairly stable protein-carbohydrate complex.

A comparison of the amino acid composition shows that human reticulin has a basic pattern similar to that of collagen. The values given for reticulin indicate lower alanine, glycine, proline, arginine and lysine ; and higher leucines, hydroxyproline and hydroxylysines. Bowes and Kenten (1949) had reported this general similarity previously, but their method of quantitative paper chromatography indicated a lower proline and hydroxyproline value of reticulin as compared to collagen. It should be noticed, however, that Bowes and Kenten (1949) used cattle material ; and Bowes *et al.* (1953) also investigated collagen from ox hide.

Further comparison of such values is not justified. Apart from the question of species difference, there is the equally important question of the homogeneity of the materials analysed as reticulin and collagen. Though we are sure of the relative histological purity of our reticulin, it is by no means certain that the collagen values are really those for a single substance. The values for collagen given in Table II are from Bowes *et al.* (1953), and these are based on those reported by Bowes and Kenten (1948), who analysed the dense dermic connective tissue of cattle as a source of collagen. These authors admit that this contains not only histological collagen, but some reticulin and elastin fibres as well. To a histologist, this analysis is of a mixture of collagen, reticulin, elastica and ground substance. Indeed, Bowes *et al.* (1953) report that after treatment of their collagen with hyaluronidase there is a slightly higher hydroxyproline, and appreciably lower tyrosine value on subsequent analysis. At the histological level, this would correspond to the removal of all, or some, of the histological ground substance.

It would seem appropriate to mention here the fallacy of drawing functional conclusions from relatively minor differences reported in analytical figures of the composition of materials analysed biochemically, unless a strict histological or histochemical control has been observed during the extraction process. The presence of 4·2 per cent of non-hexosamine carbohydrate indicates a striking difference between the composition of reticulin and collagen. Previously reported values for the carbohydrate of collagen are very low. Bangle and Alford (1954), using the anthrone reaction, found a value of 0·55 per cent carbohydrate in their collagen. They used a commercial preparation of hide powder, and it would seem that once again one may question the homogeneity of the original material. Most commercial hide powders are a mixture of denatured collagen, reticulin, elastin and ground substance, and it would be reasonable to assume that the carbohydrate value of the histologically acceptable collagen in this material would be exceedingly low, or negligible. In a previous investigation (Kramer and Windrum, 1954), the carbohydrate value of renal reticulin was given as approximately 2·5 per cent. This was estimated on a sample separated by use of decinormal sodium hydroxide, and estimated by the orcin colorimetric method (Pirie, 1936). No allowance had been made for the moisture content of the reticulin (approximately 10 per cent), and separation by use of decinormal sodium hydroxide may

remove some of the polysaccharide material from reticulin. These facts probably contribute to the difference in values reported then and now.

No uronic acid or sulphate ester was detected in this reticulin, and the sugars identified were galactose, mannose and fucose. While this work was in progress, Glegg, Eidinger and Leblond (1953) described some carbohydrate components of reticulin fibres which they isolated from cattle lymph nodes, lung, testis and adipose tissue. They showed the presence of galactose, glucose, mannose and fucose in large amounts in reticulin. These same sugars were present in only small amounts in collagen, where Glegg *et al.* (1953) considered them to represent a contamination of the collagen with other materials. These authors gave no quantitative values. We were not able to find glucose in our hydrolysates; however, it is possible that the resin method of hydrolysis used by Glegg and co-workers may have been less destructive of carbohydrate than our method of acid hydrolysis; the question of a species difference between cattle and human tissue also arises again.

Reticulin has a very low hexosamine value (0·17 per cent) and this is true also of the reported values for hexosamine in collagen. Bangle and Alford (1954) give only 0·04 per cent hexosamine in their hide powder. It is difficult to give a precise accuracy to the quantitative value of hexosamine in reticulin, because of the very small amount present, and because, as pointed out by Partridge and Gottschalk (1950), there is a possibility of an interaction arising between amino acid liberated by hydrolysis, with carbohydrate also present, to give a substance reacting like hexosamine in the estimation. However, the separation on an ion exchange resin leaves no doubt that some glucosamine is actually present, and that galactosamine is absent.

The absence of galactosamine excludes the possibility of reticulin being a complex of collagen with a "sulphate-free chondroitin" (Kramer, 1953). The absence of uronic acid would also rule out the participation of hyaluronic acid in the reticulin structure. Further, both hyaluronic acid and chondroitin sulphate have a hexose : hexosamine ratio of 1 : 1, whereas the hexose : hexosamine ratio of reticulin would be greater than 20 : 1 (including uronic acid as a hexose). The presence of galactose and mannose would not accord with the structure of any defined constituent of the ground substance, though Consden (1953) has drawn attention to their occurrence in subcutaneous tissue and in synovial fluid. Fucose is a common constituent of epithelial mucins (Werner, 1953) and has been described in reticulin by Glegg *et al.* (1953). In our chromatograms, galactose and mannose seemed to be present in greater amounts than fucose.

It is interesting to compare the composition of this reticulin with lens capsule. Lens capsule has 10 per cent carbohydrate (mostly glucose and some galactose) and an insoluble protein, digestible by collagenase and having an amino acid composition similar to that of collagen by chromatographic analysis (Pirie, 1951). Hexosamine is present to the extent of approximately 1 per cent, so that here we also have an example of a mucosubstance which has very little hexosamine in relation to total carbohydrate.

The presence of such a large lipid fraction in the reticulin was the most surprising finding of these analyses. When it first appeared, it seemed reasonable to assume that it was a contaminant, but this possibility was minimised since the lipid is liberated (as fatty acid) only after hydrolysis.

It is unlikely that the lipid fraction arose from contaminating cellular lipo-

proteins since tyrosine was present to such a small extent in our material. Lipid fractions of human reticulin have been described previously (Foot, 1928), though it seems probable that these were of cellular origin, since they were extractable from the intact unhydrolysed reticulin preparation by organic solvents, and were composed of lethicin, cholesterol, fats and fatty acids. In our work the lipid fraction did not appear until after hydrolysis of the material left after prolonged Soxhlet extraction.

An investigation of the X-ray diffraction pattern of reticulin, collagen and this lipid material (Little and Windrum, 1954) suggests that the difference in the X-ray diffraction pattern of reticulin and collagen may actually be due to the presence of this lipid material in the reticulin, but not in collagen. The X-ray diffraction pattern of reticulin has previously been reported by Kramer and Little (1953) to show the characteristic collagen spacings, with additional diffraction rings that they had attributed to the presence of amorphous material. Little and Windrum (1954) have shown that these additional rings are due to the fatty acid in reticulin.

Thus, in our view, reticulin is composed of conjugated protein, carbohydrate and lipid constituents and, in this respect, is markedly different from collagen.

These observed differences in chemical composition of reticulin and collagen allow a rational approach to be made to the problem of their different reactions to histological and histochemical methods.

The common empirical methods for reticulin and collagen, such as Mallory's connective tissue stain, show a close similarity ; this is probably attributable to the marked similarity of the main protein constituents of both materials. The differing reaction of reticulin from that of collagen to the P.A.S. and sulphation-metachromasia reactions seems to be adequately explained by the fact that reticulin contains an appreciably greater quantity of carbohydrate than does collagen.

Wolman (1950) suggested that the P.A.S. reaction of reticulin was not due to carbohydrate but to the presence of an unsaturated fatty acid, possibly of the sphingolipid type. He based this assumption on his failure to block the P.A.S. reaction of reticulin by an acetylating procedure. Lillie (1951) has drawn attention to the inadequacy of such acetylating techniques, and shown that more adequate acetylation will block the P.A.S. reaction of reticulin. This observation has been confirmed, and similarly acetylation will block the sulphation-metachromasia reaction, if adequately done (Kramer and Windrum, 1954). However, to ensure that the lipid does not contribute to the P.A.S. reaction of reticulin, some of the lipid material extracted from reticulin was smeared on glass slides and subjected to the P.A.S. technique (except, of course, that the slide was examined in water at the end, since dehydrating in alcohol would immediately dissolve the material). No staining resulted, even if the period of exposure to periodic acid was increased to 1 hr., and so it seemed certain that the presence of the lipid did not contribute to the P.A.S. reaction.

An additional histochemical method of distinguishing reticulin and basement membranes from collagen has been described by Cruickshank and Hill (1953), who showed that an antigenic property resides in these materials. By use of a fluorescent antibody technique, developed from that of Coons, Creech, Jones and Berliner (1942) and Coons and Kaplan (1950), they showed that a common antigen is present in epithelial basement membrane, reticulin, sarcolemma, and neurilemma, but absent from collagen, cartilage and elastic tissue. This difference in immunological reaction may be due to the presence of carbohydrate in reticulin,

as it is known that antigenic properties may depend on the presence of polysaccharides. It is interesting to see that there is no cross-immunological reaction with cartilage, as the sugars isolated from reticulin are different from those of chondroitin sulphate, which is considered to be the main carbohydrate present in cartilage. Indeed, Cruickshank and Hill (1953) do attribute this property to a polysaccharide, and comment on the lack of response of hyaluronic acid and cartilage.

The lipid fraction may be responsible for some of the properties of isolated reticulin described by Kramer and Little (1953). The stability of reticulin to boiling water, the fact that it will withstand several hours' boiling in normal hydrochloric acid, may thus be explained; more significant is the fact that it is readily soluble in boiling sodium hydroxide, which could be explained by the saponification of the lipid present, converting it to a readily soluble soap.

This lipid fraction may also be of considerable significance in pathological processes, particularly in regard to the appearance of lipid in degenerate " hyalinised " connective tissue. The appearance of free stainable lipid is often a very early feature of hyaline in many situations, including the early lesions of atherosclerosis. It may be that a failure of the normal factors (at the moment largely unknown) responsible for the maintenance of reticulin and basement membranes, including the intima of vessels, may be revealed by an unmasking of this bound lipid in these areas. (Rinehart (1930) considered that vessel intima was typical reticulin.) Faber (1949) and Baker and Selikoff (1952) have drawn attention to the fact that the lipid of atheromatous lesions, of large vessels and intimate renal vasculature, is not only cholesterol and its derivatives, but contains other lipids as well. Taylor (1953) has noted the early accumulation of small streaks of lipid material in vessel walls showing slight alteration in the intimal mucopolysaccharides, and that an intimate admixture of such lipid and mucopolysaccharide is often one of the earliest signs of hyalinisation. Though most authors, including Taylor (1953), assume that this lipid is derived from the blood stream, it would seem that the finding of such a large amount of lipid bound to reticulin may suggest the lipid is actually released from degenerate reticulin and intimal walls.

Another question which is raised by the finding of this lipid fraction in renal reticulin is whether the reticulin of such parenchymatous organs is, in fact, the same substance as the precollagenous reticulin seen in sclerosing processes. This assumption is mainly supported by their similar histochemical and histological reaction. Of these, the mechanism of the silver reaction is ill understood; the P.A.S. and sulphation-metachromasia reactions depend on the presence of carbohydrate, and would be given equally well by a lipo-glyco-protein (renal reticulin), or an intermediatory glycocollagen complex, which may be the actual reticulin of sclerosing processes. Available histochemical techniques are not specific enough to distinguish between these two presumed types of material. The reticulin of sclerosing processes may actually have a quite different type of carbohydrate fraction from that of renal reticulin. Perhaps the method of Cruickshank and Hill (1953) could be used to see if there is an immuno-chemical similarity of sclerosing reticulin and organ reticulin. This method seems at present to be the only one available that would supply the answer, or part of it, since methods of extraction would apparently not serve.

The fluorescent antibody technique of Cruickshank and Hill (1953) showed the presence in epithelial basement membranes and reticulin of a common antigen

not present in collagen and elastic tissue. While most histologists consider that reticulin and basement membranes are similar, others regard them as separate structures. Thus Gersh and Catchpole (1949) consider basement membranes as a condensation of ground substance and distinct from reticulin. Previous workers, such as Rössle and Yoshida (1909) and Foot and Day (1925), definitely identified the basement membranes of various glands with the argyrophilic reticulin. Plenck (1927) recognised the presence of hyaline membranes distinct from the abutting reticulin only in the kidney about the tubules, and around sebaceous glands.

Although the silver impregnation and the sulphation-metachromasia methods can both be used to demonstrate basement membranes and reticulin, they do not give exactly the same picture. The former shows basement membranes as finely fibrillar structures whereas the latter makes them appear as homogeneous membranes. This difference can be clearly seen if the silver impregnation be followed by the sulphation-metachromasia reaction on the same section; basement membranes then appear as fine black fibrillar structures closely outlined by a thin layer of a homogeneous red-staining material.

As a result of their extensive electron microscopic study, Kramer and Little (1953) concluded that reticulin and basement membranes were essentially similar, consisting of fine randomly arranged fibrils lying in an apparently amorphous matrix. They found material agreeing in all respects with renal cortical tubular basement membrane in spleen, lymph nodes, myocardium, liver, adrenal and other organs. They also noted that the relative proportions of fibrils and amorphous material varied considerably in different samples, even in material from the same source. Hence it is well to emphasise that the chemical analyses reported here are of the renal cortical reticulin from subjects in the age group 15–25 years, and that there may be considerable variation in the composition of reticulin from different organs and at different ages.

SUMMARY.

Renal cortical reticulin is composed of approximately 85 per cent protein, having an amino acid composition similar to collagen. Nineteen amino and imino acids have been identified. Carbohydrate is present to the extent of 4·2 per cent non-hexosamine, and the hexosamine content is very low. The remainder of the material (10·9 per cent) is composed of bound fatty acids, predominantly myristic acid. The presence of the carbohydrate fraction offers an explanation for some of the distinctive histochemical properties of reticulin. The lipid may be of significance in degenerative processes in connective tissue.

REFERENCES.

Baker, R. D. and Selikoff, B.—(1952) *Amer. J. Path.*, **28**, 573.
Bangle, R. and Alford, W. C.—(1954) *J. Histochem. Cytochem.*, **2**, 62.
Bielschowasky, M.—(1905) *Arch. Psychiat. Nervenkr.*, **39**, 1321.
Bowes, J. H., Elliot, R. C. and Moss, J. A.—(1953) 'Nature and Structure of Collagen.' London (Butterworth Scientific Publications).
Idem and Kenten, R. H.—(1948) *Biochem. J.*, **43**, 350.—(1949) *Ibid.*, **45**, 281.
Consden, R.—(1953) 'Nature and Structure of Collagen.' London (Butterworth Scientific Publications).
Idem, Gordon, A. H. and Martin, A. J. P.—(1944) *Biochem. J.*, **38**, 224.

COONS, A. H., CREECH, H. J., JONES, R. N. AND BERLINER, E.—(1942) *J. Immunol.*, **45**, 199.
Idem AND KAPLAN, H. H.—(1950) *J. exp. Med.*, **91**, 1.
CROPPER, F. R. AND HEYWOOD, A.—(1953) *Nature, Lond.*, **172**, 1101.
CRUICKSHANK, B. AND HILL, A. G. S.—(1953) 'Nature and Structure of Collagen.' London (Butterworth Scientific Publications), p. 27
DREYWOOD, R.—(1946) *Industr. Engng Chem.* (*Anal.*), **18**, 499.
EASTOE, J. E.—(1954) *Nature, Lond.*, **173**, 540.
Idem AND EASTOE, B.—(1954) *Biochem. J.*, **57**, 453.
ELSON, L. A. AND MORGAN, W. T. J.—(1933) *Ibid.*, **27**, 1824.
FABER, H.—(1949) *Arch. Path.*, **48**, 342.
FOOT, N. C.—(1928) *Amer. J. Path.*, **4**, 523.
Idem AND DAY, H. A.—(1925) *Ibid.*, **1**, 431.
GARDEL, S.—(1953) *Acta chem. scand.*, **7**, 207.
GERSH, I. AND CATCHPOLE, H. R.—(1949) *Amer. J. Anat.*, **85**, 457.
GLEGG, R. E., EIDINGER, D. AND LEBLOND, C. P.—(1953) *Science*, **118**, 614.
HOTCHKISS, R. D.—(1948) *Arch. Biochem.*, **16**, 131.
KRAMER, H.—(1953) 'Nature and Structure of Collagen.' London (Butterworth Scientific Publications). In discussion, p. 47.
Idem AND LITTLE, K.—(1953) 'Nature and Structure of Collagen.' London (Butterworth Scientific Publications), p. 33.
Idem AND WINDRUM, G. M.—(1953) *J. clin. Path.*, **6**, 239.—(1954) *J. Histochem. Cytochem.*, **2**, 196.
KUPFFER, C.—(1876) *Arch. mikr. Anat.*, **12**, 353.
LILLIE, R. D.—(1947) *J. Lab. clin. Med.*, **32**, 910.—(1951) *Stain Tech.*, **26**, 123.—(1953) *J. Histochem. Cytochem.*, **1**, 353.
LITTLE, K. AND WINDRUM, G. M.—(1954) *Nature, Lond.*, in press.
MACFARLANE, R. G. AND MACLENNAN, J. D.—(1945) *Lancet*, **2**, 328.
MALL, F. P.—(1888) *Anat. Anz.*, **3**, 397.—(1896) *Johns Hopk. Hosp. Rep.*, **1**, 171.
MALLORY, F. B. AND PARKER, F.—(1927) *Amer. J. Path.*, **3**, 515.
MARESCH, R.—(1905) *Zbl. allg. Path. path. Anat.*, **16**, 641.
MCMANUS, J. F. A.—(1946) *Nature, Lond.*, **158**, 335.
MOORE, S. AND STEIN, W. H.—(1948) *J. biol. Chem.*, **176**, 367.—(1951) *Ibid.*, **192**, 663.
NEUMAN, R. E. AND LOGAN, M. A.—(1950) *Ibid.*, **184**, 299.
PARTRIDGE, S. M.—(1949) *Nature, Lond.*, **161**, 443.
Idem AND GOTTSCHALK, A.—(1950) *Ibid.*, **165**, 684.
PEARSE, A. G. E.—(1953) 'Histochemistry—Theoretical and Applied.' London (J. & A. Churchill Ltd.).
PIRIE, A.—(1951) *Biochem. J.*, **48**, 360.
PIRIE, N. W.—(1936) *Brit. J. exp. Path.*, **17**, 274.
PLENCK, H.—(1927) *Z. ges. Anat.* **3**. *Ergebn. Anat. EntwGesch.*, **27**, 302.
RINEHART, J. F.—(1930) *Amer. J. Path.*, **6**, 525.
ROBB-SMITH, A. H. T.—(1945) *Lancet*, **2**, 362.—(1952) 'Connective Tissues,' Trans. 3rd Conf., Josiah Macy Jr. Foundation, New York.
RÖSSLE, R. AND YOSHIDA, T.—(1909) *Beitr. path. Anat.*, **45**, 110.
SCHRAM, E., DUSTIN, J. P., MOORE, S. AND BIGWOOD, E. J.—(1953) *Analyt. chim. acta*, **9**, 149.
TAYLOR, H. E.—(1953) *Amer. J. Path.*, **29**, 871.
TOLLENS, B.—(1908) *Ber. dtsch. chem. Ges.*, **41**, 1788.
WERNER, I.—(1953) *Acta Soc. med. Uppsaliensis*, **58**, 1.
WOLMAN, M.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **75**, 583.

BRUCELLA SUIS INFECTION OF GUINEA-PIGS BY THE RESPIRATORY ROUTE.

G. J. HARPER.

From the Microbiological Research Department, Ministry of Supply, Porton, Wilts.

Received for publication October 5, 1954.

THERE is no evidence to show that *Brucella suis* either injected into or inhaled by a susceptible animal produces any early pathological change in lung tissue. On the other hand the organism is highly infectious when inhaled ; an estimated inhaled dose of about thirty viable cells produces a fifty per cent chance of infection in the guinea-pig (Elberg and Henderson, 1948). A constant and very early pathological change shown by such animals is enlargement of the bronchial or cervical lymph nodes, according to the size of the particle in which the organisms are presented to the host (Druett, Henderson and Peacock, unpublished work) ; the lymph nodes also show massive invasion by *Br. suis*.

In the light of these findings it seemed possible that the organism deposited on the alveolar surface was capable of multiplying thereon without penetrating lung tissue to produce pathological change in it. If this were so then as numbers increased so too would the chance of one or more cells being phagocytosed and transported to a nearby lymph node. This site could then be that from which there was a welling over of organisms into the circulation to produce generalised infection.

The experiments described in this paper are primarily concerned with an examination of this hypothesis.

MATERIALS AND METHODS.

Preparation of infecting suspension of Br. suis.

Dried cultures of *Br. suis* (Strain PS III) were taken up in Zobell's (Zobell and Zobell, 1932) fluid and placed on slopes of fortified tryptose agar (F.T.A.) (McCullough, Mills, Herbst, Roessler and Brewer, 1947). The 48-hr. growth from one slope was used to inoculate a larger slope (8-oz. (226·8 g.) bottle containing 30 ml. F.T.A.). After 48 hr. incubation at 37° the resulting growth was washed off with 25 ml. Zobell's fluid, the suspension examined for purity, and viable counts carried out. A fresh batch of suspension was prepared for each experiment.

Animals.—Guinea-pigs of 400–500 g. weight from a healthy stock were used.

Method of infecting animals.—Animals were exposed to a measured concentration of a single-cell aerosol of *Br. suis* in the Henderson (1952) apparatus for 1 min. The animals were housed 5 in a cage.

Post-mortem procedure.

Guinea-pigs were killed and the following examination made.

Body weight determined ; heart blood removed for culture and serology ; the appearance of the organs noted ; bone marrow, liver, spleen, bronchial and cervical lymph nodes and lungs cultured ; spleen and lungs removed to sterile 1-oz. bottles for viable counts ; bronchial lymph nodes removed for grinding and culture (Exp. 3 only).

Direct culture of tissues.

Tissues, with the exception of bone marrow, were cultured by rubbing a cut surface over half a plate of F.T.A. to which was added 1 part in 8 million brilliant green and 4 units/ml. polymyxin (Burroughs Wellcome) to prevent overgrowth with contaminants encountered in undiluted lung samples.

Viable counts on lungs and spleen.

Tissue weights were measured and Zobell's fluid added. For the purpose of determining total volumes of tissue suspensions the S.G. of tissue was taken as 1·0. Spleens were made up to 4, 5, 10 or 15 ml. according to spleen weight and expected count. Lungs were suspended in 10 ml. of fluid.

The tissues were disintegrated for 2 min. in specially designed high-speed emulsifiers. Preliminary test had shown this period to be satisfactory and to have no effect on the viability of dilute suspensions of *Br. suis*. Spleens emulsified well. Lungs needed a preliminary mincing with scissors; the tissue was not as completely broken down as spleen tissue, but made workable suspensions.

Tissue suspensions were diluted, in tenfold steps, in 0·1 per cent tryptose saline, and 0·2 ml. volumes spread over the surface of F.T.A. plates. Duplicate or triplicate plates were used for each dilution.

Grinding of bronchial lymph nodes.

In one experiment two or more bronchial lymph nodes from each animal were removed to a sterile 3 in. × ⅜ in. (7·62 × 0·95 cm.) test-tube containing a layer of washed sterile sand, and were broken up by grinding with a sterile glass rod after adding 0·4 ml. Zobell's fluid. The samples were cultured by direct plating of the bulk of the suspension and adding fortified tryptose broth to the remainder. The broth cultures were plated on F.T.A. after 3, 7, 14 and 21 days' incubation at 37°.

Blood cultures.

By means of capillary pipettes 2 to 3 ml. of heart blood were collected and added to 20 ml. fortified tryptose broth containing 1 part in 12 million of brilliant green. (The growth from an inoculum of 42 cells of *Br. suis* had been tested in this medium and found satisfactory.) Cultures were incubated at 37° and subcultured on F.T.A. after 3, 7, 14 and 21 days' incubation.

From some animals quantitative blood cultures were made by inoculating F.T.A. plates with 1/12th ml. of blood, spreading, and incubating at 37°. The blood counts were expressed as viable cells/ml.

Marrow cultures.—The muscles were dissected from the right femur, the bone broken with bone forceps, and a sterile loop passed into the medullary cavity. The marrow removed was spread over a plate of F.T.A.

Serology.

Agglutination tests were done with serum from test animals separated from heart blood collected in dry sterile tubes. Twofold dilutions from 1/5–1/5120 were mixed with equal volumes of *Br. suis* suspension and the results read after 24 hr. in a water bath at 56°. The heat-killed suspension of the infecting strain was diluted to opacity 3 (Brown's Scale).

Identification of Br. suis.—All cultures presumed to be *Br. suis* by colonial appearance were confirmed morphologically and by slide agglutination.

RESULTS.

Three experiments were made and in each a group of 100 guinea-pigs was exposed. The dosage was varied to meet the needs of each test and Table I shows the details. The estimated intake of *Br. suis* is based on a respiratory minute volume of 150 ml. and the lung retention is taken as 50 per cent of this value (Harper and Morton, 1953).

In recording results, quantitative data are given only for lung tissue. The more qualitative method of direct plate culture was used as an indicator of the path

of infection to other tissues. To examine the validity of this procedure quantitative data available from lung and spleen viable counts were compared with the qualitative results of testing these tissues. When the viable count reached about 10^4 cells per g. of tissue, lung or spleen, then only occasional colonies were

TABLE I.—*Dosage of* Br. suis *Used in Experiments* 1, 2 *and* 3.

Experiment number.	Cloud concentrations (cells/litre).	Estimated respiratory intake.	Estimated lung retention.	Calculated* ID.
1	450	68	34	80%
2	3,110	467	234	100%
3	774	116	58	90%

* From regression line data of Elberg and Henderson (1948) and unpublished experiments in this Department.

observed by the direct plate method. When the count in these tissues reached about 5×10^5 per g. of tissue, a confluent growth was obtained by the direct plate method. It follows that a negative finding obtained by this latter method is not proof of absence of infection. Nevertheless the method gives useful guidance to the degree and the route of infection. For example, if positive bronchial lymph nodes are found in a series of animals in which all tissues other than lung are negative, then we may reasonably assume that at least one route leading to generalised infection is *viâ* these lymphatic glands.

Development of Generalised Infection.

Experiment 1.—One hundred animals were exposed to a cloud concentration expected to produce about 80 per cent of infected animals within 28 days. Through accident and non-specific deaths only 93 remained for examination. Fifty-five animals were killed in groups of 5 at short intervals from 4 to 28 days after exposure to find the most profitable time for a more intensive examination of the initiation of the disease. The remaining animals were killed at later stages to examine the progress of the established disease. The results are given in Table II. The bacterial counts are expressed as the arithmetic mean in each group. There was, however, a wide scatter around the mean (see Table V).

The first three lots of animals examined between 4 and 11 days after exposure showed a marked increase in the number of *Br. suis* in lung tissue over the few organisms originally retained. While this occurred, there was no infection detected by the direct plate method in the regional lymph nodes, spleen or liver. The first evidence of infection in these organs occurred on the 13th day, by which time the viable count in the lungs had risen to $2–3 \times 10^4$—an increase of about 1000-fold over the number originally inhaled and retained. After the infection had reached the bronchial lymph nodes the spread to other tissues was rapid, and the animals developed typical lesions which were well established by the 19th day. These preliminary findings helped to plan a second experiment designed to give more information about the early stages of the disease.

Experiment 2.—Animals in this group were exposed to a dose giving an estimated lung retention of 250 cells, which would be expected to infect about 100 per cent of guinea-pigs. The animals were sacrificed in groups of 10 at 2-day intervals.

Table III shows that the same pattern of events occurred as in the first experiment, but that the whole process is accelerated. The gross pathology was typical in all animals killed on or after the 14th day. The possibility that lightly infected bronchial glands were not being detected by direct plate culture led to the third experiment.

TABLE II.—*Experiment* 1: *Results of Exposure of* 93 *Guinea-pigs to a Cloud of* Br. suis (*Estimated Lung Retention* 34 *Cells* $\equiv ID_{80}$). *Animals Killed in Groups of* 5.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$	Direct plates, number positive. Bronchial.	Cervical.	Spleen.	Liver.	Bone marrow.	Blood culture. Number positive.	Blood culture. Count/ml.	Agglutinins. Number positive.	Agglutinins. Titre.
4	1	0·44	0	0	0	0	0	0	—	0	—
7	2	0·12	0	0	0	0	0	0	—	0	—
11	4	0·95	0	0	0	0	0	1	—	0	—
13	5	2·7	5	2	1	1	0	1	—	0	—
15	5	80·8	2	1	2	2	2	2	—	2	180
17	3	68·3	3	1	1	1	1	2	—	1	320
19	5	52·4	3	3	3	3	3	3	—	3	213
21	5	250·5	5	5	5	5	5	5	—	5	512
24	4	101·2	4	4	4	4	4	4	—	4	1280
26	4	367·0	4	4	4	4	4	4	—	4	560
28	5	732·0	5	5	5	5	5	5	—	5	960
35	4	60·0	4	4	4	4	4	3	93	4	2080
42	2	41·9	2	2	2	1	1	2	6	2	5120
56	4	39·9	4	5	3	1	0	2	0	5	2816
84	5	141·2	2	2	2	1	1	2	48	5	2240
96	5	2060·0	3	3	1	0	0	0	—	5	2040
126	5	1008·0	3	2	1	0	0	0	—	5	1536
154*	2	121·0	1	0	1	0	0	0	—	4	1920
215*	2	1350·0	1	0	0	0	0	0	—	4	1280

* Groups of 4 animals killed at these times.

In this and succeeding Tables mean viable counts are per organ.

TABLE III.—*Experiment* 2: *Results of Exposure of* 100 *Guinea-pigs to a Cloud of* Br. suis. (*Estimated Lung Retention* 234 *Cells* $\equiv ID_{100}$). *Animals Killed in Groups of* 10.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$.	Direct plates number positive. Bronchial.	Cervical.	Spleen.	Liver.	Bone marrow.	Blood culture. Number positive.	Blood culture. Count/ml.	Agglutinins. Number positive.	Agglutinins. Titre.
2	9/9*	0·09	0	0	0	0	0	0	—	0	—
4	8/9*	1·84	0	0	0	0	0	0	—	0	—
6	10	12·3	3	0	0	0	0	0	—	0	—
8	9/9*	38·1	6	2	1	0	0	1	<12	0	—
10	10	24·9	9	6	4	3	0	4	<12	0	—
12	10	40·2	8	3	7	4	1	6	<12	1	10
14	9	157·2	9	5	8	6	7	9	340	5	224
16	10	106·0	9	9	9	9	9	9	512	9	446
18	10	290·0	10	9	10	10	10	10	806	10	356
20	10	209·0	10	10	10	10	10	10	898	10	584

* Cultures lost due to contaminations.

Experiment 3.—Animals were exposed to a dose giving an estimated lung retention of about 60 cells and were examined in groups of 10 at daily intervals commencing 24 hr. after exposure. In this series special attention was paid to the bronchial lymph nodes, which were cultured by a method designed to detect small numbers of *Br. suis* (see Materials and Methods).

Tables IV and V show that *Br. suis* multiplies in the lungs before infection of the bronchial lymph nodes can be detected by the most sensitive culture method. It will be noted that in some of the animals examined on the 7th and 9th days after infection growth of *Br. suis* was obtained from the cervical lymph nodes when the bronchial nodes were sterile ; one animal had a lung viable count of only 91 cells. All the cultures of spleen suspensions made on the 9th day were sterile and the lung viable count showed a marked fall-off. No explanation can be found for these results which are contrary to the findings in Exp. 1 and 2. Technical error cannot positively be ruled out.

It is of interest that of the 23 bronchial lymph node samples which gave positive cultures 16 were detected by direct plating, 21 by plating of tissue suspensions and 23 by the fluid culture technique.

Progress of the Established Disease.

Animals surviving after the 28th day in Exp. 1 were used to observe the progress of the established disease. The results are shown in Table II. There were no deaths in this group. Animals examined in the early stages of the disease looked emaciated and had lost weight ; those examined later looked well and put on about the same amount of weight as would normal guinea-pigs under similar conditions.

Between the 35th and 84th day lung viable counts were about 1/10th of the peak value reached on the 28th day. Tests on the 96th day showed a sharp rise in counts which persisted to the 215th day. This was associated with lung abscesses found in animals killed at these times.

Between 35 and 215 days after infection the spleen viable count showed a decline to about 0·1 per cent of the peak value recorded on the 28th day (see Table VI). The spleen suspension was the only tissue that was positive in all the 4 animals examined on the 215th day. Two animals had positive lung cultures and one a scanty infection in the bronchial lymph nodes at this time. Cultures made from the cut surface of spleens were sterile.

A change in the character of the lesions was noted in animals held for 56 days or longer. There was then a marked tendency for the cervical lymph nodes to contain numerous small abscesses, which later coalesced to form one large abscess filled with thick creamy pus. Spleens showed a few large abscesses in contrast to the more numerous small ones seen in animals examined earlier. The liver (except in one animal killed on the 154th day, which contained one large abscess) showed shallow pits over the surface corresponding in distribution with the small abscesses seen in animals killed earlier in the disease. The spleen size, in proportion to the body weight, showed a decline in the later stages of the disease, but after 215 days was still twice the normal size. Lungs measured on the same basis showed little variation except when abscesses were present late in the disease ; such lungs were 2–3 times the normal weight.

TABLE IV.—*Experiment* 3: *Results of Exposure of* 100 *Guinea-pigs to a Cloud of* Br. suis. (*Estimated Lung Retention* 58 *Cells* $\equiv ID_{90}$). *Animals Killed in Groups of* 10.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$	Direct plates number positive. Bronchial.*	Cervical.	Spleen.	Liver.
1	6	0·002	0	0	0	0
2	9	0·041	0	0	0	0
3	6	0·035	0	0	0	0
4	7	0·78	1	0	0	0
5	7	0·77	2	1	0	0
6	9	5·70	1	1	0	0
7	10	8·29	2	3	0	0
8	9	36·6	6	0	0	0
9	6	2·50	4	2	0	0
10	9	7·0	7	3	2	2

* Includes positive cultures from emulsified lymph nodes and broth cultures.

TABLE V.—*Experiment* 3: *Lung Viable Counts in* 100 *Guinea-pigs Exposed to a Cloud of* Br. suis. (*Estimated Lung Retention* 58 *Cells*). *Animals Killed in Groups of* 10.

Days after exposure.	Lung viable counts $\times 10^{-3}$				
1	0 0·1	0 0·1	0 0·2	0 0·3	0·1 0·4
2	0 2·5	0·7 4·1	1·2 4·9	1·2 5·1	1·6 9·6
3	0 0·6	0 0·6	0 2·3	0 7·9	0·4 9·0
4	0 13·3	0 21·2	0 43·0	0·8 **47·8**	10·7 **413·0**
5	0 25·4	0 27·6	0 **56·0**	2·5 71·9	18·7 **334·0**
6	0 57·7	1·0 62·1	27·1 74·0	27·6 148·0	29·4 **3650**
7	0·9* 75·4*	6·7 113·0	8·3 139·0*	27·8 221·0	**75·1** **7620**
8	0 **368·0**	9·5 716·0	27·5 **7220**	**30·1** **12,100**	**97·6** **12,700**
9	0 **72·2**	0 91·7	0 **138·0**	0 **303·0**	**12·7** 895·0*
10	0 **78·2**	**18·8** **145·0**	24·0 **382·0**	**67·9** **1600**	77·6 **3800**

Heavy type = Bronchial glands yield growth of *Br. suis*.

* Cervical glands yield growth of *Br. suis*; bronchial glands negative.

TABLE VI.—*Spleen Viable Counts Experiment* 1.

Days after exposure.	Viable count $\times 10^{-4}$.
4	0
11	0·57
15	956·0
19	600·0
24	845·0
28	3180·0
42	196·0
84	388·0
126	0·54
215	2·02

Cultures made from animals examined later than the 42nd day showed a fall-off in growth on the direct plates and by the 154th day the majority of plates made from tissues showing typical lesions were sterile. Victor and Valliant (1952) also noted a fall-off in positive cultures made from tissues containing lesions in animals held 15–20 weeks after infection with *Br. suis*.

One animal examined 84 days after exposure was in marked contrast to the other animals killed at the same time. The tissue appearance and cultural findings were consistent with an infection of shorter duration (including positive blood and marrow cultures). This animal may have had a much longer incubation period than its fellows or may have been cross-infected from animals put in the same room 28 days later. The latter possibility seems slight as we have no evidence of cross-infection amongst animals exposed to a single cell aerosol of *Br. suis*.

Blood and Bone Marrow Cultures.

Cultures were made from all animals in Exp. 1 and 2; quantitative blood cultures were made on animals examined on and after the 35th day in Exp. 1 and on all animals in Exp. 2. The results are shown in Fig. 1 and 2. A positive blood picture appeared from the 11th day in Exp. 1 and from the 8th day in 2 (where the dosage was higher). The proportion of infected animals yielding positive blood cultures reached 100 per cent on the 21st and 14th days respectively. By the 96th day the blood was sterile although tissues were still yielding positive cultures. The degree of infection in the blood rose from less than 12 cells/ml. on the 8th day to a mean count of about 900/ml. on the 20th day. (Individual animals had as many as 2000 ml. on the 18th day.) By the 56th day counts had again fallen to less than 12/ml. The one higher value obtained on the 84th day was associated with the anomalous results already referred to in the last section.

The bone marrow cultures followed the same trend as the blood cultures but in both experiments positive results were not obtained until the blood had been positive for 4 days. As with the blood, the marrow was sterile by the 96th day. The method used for culturing blood was more sensitive and it may be that the direct plating of marrow did not detect all infected samples.

Agglutinins.

Sera from all animals in the first two experiments were examined for the presence of specific agglutinins. The results are shown in Fig. 3 and 4. The presence of agglutinins was first noted on the 15th day in Exp. 1 and on the 12th day in 2.

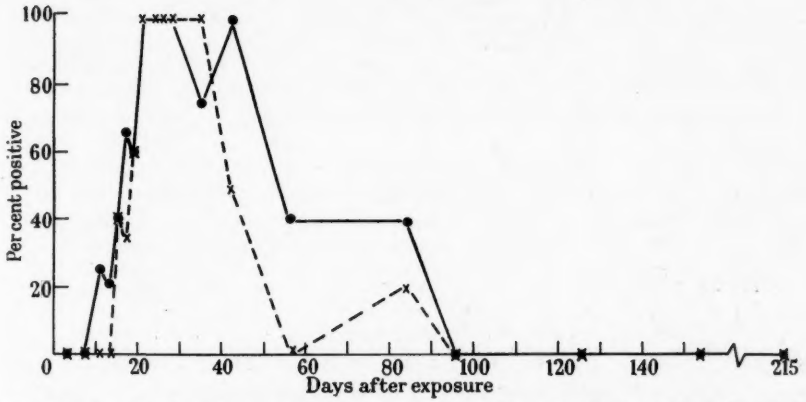


FIG. 1.—Exp. 1. Blood and marrow cultures: per cent positive of animals yielding *Br. suis* from other tissues.

● ———— ● = Blood culture.
×— — — —× = Marrow culture.

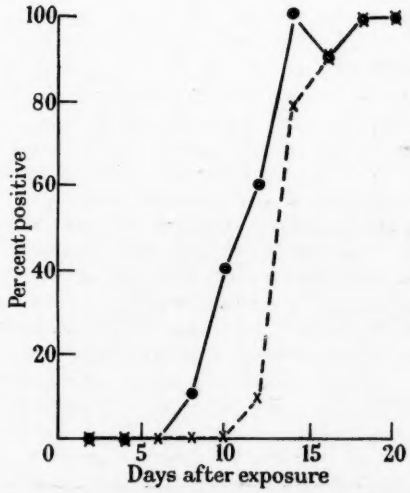


FIG. 2.—Exp. 2. Blood and marrow cultures: per cent positive of animals yielding *Br. suis* from other tissues.

● ———— ● = Blood culture.
×— — — —× = Marrow culture.

FIG. 3.—Exp. 1. Agglutinins.

● ——— ● = Titre.
×— — — —× = Per cent positive.

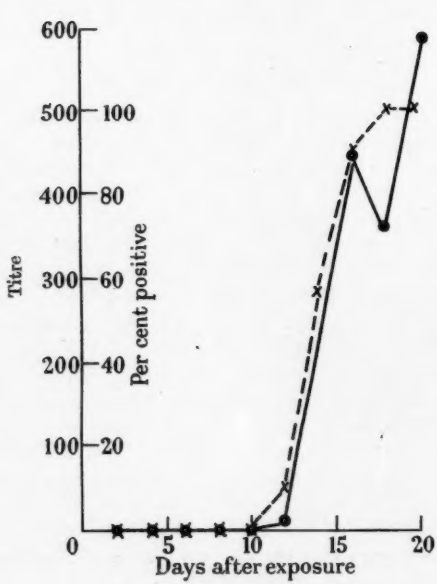


FIG. 4.—Exp. 2. Agglutinins.

● ——— ● = Titre.
×— — — —× = Per cent positive.

The proportion of infected animals showing agglutinins reached 100 per cent on the 21st and 18th days respectively. The titre rose to a peak of 5120 after 42 days and then slowly declined, but was still 1280 after 215 days. The rises in titre in the first 20 days in the two experiments were roughly parallel, the rise commencing earlier in the second (where the dosage was higher). Throughout the remainder of the 215-day period all infected animals retained agglutinins in their sera.

In all animals with a titre of 80 or over there was evidence of splenic enlargement and heavy infection. Positive sera were obtained only from animals with evidence of splenic infection. In the developing disease the threshold for obtaining a positive serological reaction is the presence of 10^6 bacterial cells/g. of spleen tissue and a spleen weight of about 0·24 per cent of the body weight (twice the normal spleen size).

DISCUSSION.

The experiments offer clear evidence of the multiplication of *Br. suis* retained in the lungs of guinea-pigs exposed to a single-cell aerosol. Initially, as one might expect, the rate of increase in numbers of viable cells is dependent on the size of the retained dose ; when a concentration of 10^3 viable cells/g. of lung tissue is reached the subsequent rate of increase is independent of the size of the retained dose. Before any other tissues can be shown to be infected the lung viable count has risen to about 300 times the respiratory ID_{50}. The order of infection of other tissues suggests that the spread is *viâ* the regional lymph nodes and blood stream. Once infection can be demonstrated in regional lymph nodes the spread throughout the body is rapid.

These findings support the hypothesis, put forward by Elberg and Henderson (1948), that *Br. suis* retained in the lungs does not give rise to demonstrable lesions but multiplies at the site of deposition until phagocytosis takes place and viable brucella are transported to the nearby lymph nodes. From this site infection of the blood stream takes place which leads to generalised infection. Macroscopic lung lesions were only seen in animals held for 96 days or longer and were one of the features of the later stages of the disease.

Once established the disease becomes chronic, and although the blood and marrow become sterile by the 96th day after exposure, and the numbers of viable brucella in the tissues decline, after 215 days the spleen and some of the other tissues are still enlarged, contain abscesses and yield viable brucella. These findings are in contrast with those of de Ropp (1945) who found with guinea-pigs infected with large numbers of *Brucella abortus* that although agglutinins were still present in the blood, the spleen was sterile and had returned to normal size 30 weeks after infection. It would appear that guinea-pigs can rid themselves more readily of *Br. abortus* than of *Br. suis*.

SUMMARY.

Multiplication of *Br. suis* in the lungs of guinea-pigs exposed by the respiratory route to small numbers of organisms has been shown to be actively in progress as early as 48 hr. after the exposure. In the absence of any evidence of invasion of lung tissue the findings are in keeping with the hypothesis that the multiplication takes place on the surface of lung epithelium and that organisms are transported by phagocytes to lymph nodes from which sites the infection becomes generalised.

Observations on the persistence of *Br. suis* (Strain PS III) in the infected animal showed that tissue was still actively infected 215 days after exposure by the respiratory route to a minimal infecting dose.

I am indebted to Dr. D. W. Henderson and Mr. J. D. Morton for constant advice and criticism during the progress of the work and preparation of this paper. Thanks are also due to Mrs. J. Saunders for technical assistance.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this work.

REFERENCES.

ELBERG, S. S. AND HENDERSON, D. W.—(1948) *J. infect. Dis.*, **82**, 302.
HARPER, G. J. AND MORTON, J. D.—(1953) *J. Hyg., Camb.*, **51**, 372.
HENDERSON, D. W.—(1952) *Ibid.*, **50**, 53.
MCCULLOUGH, W. G., MILLS, R. C., HERBST, E. J., ROESSLER, W. G. AND BREWER, C. R.—(1947) *J. Bact.*, **53**, 5.
DE ROPP, R. S.—(1945) *J. comp. Path.*, **55**, 70.
VICTOR, J. AND VALLIANT, J. R.—(1952).—*J. infect. Dis.*, **91**, 207.
ZOBELL, C. E. AND ZOBELL, M. H.—(1932) *Ibid.*, **50**, 538.

ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION.

I. AN ACTIVABLE PERMEABILITY FACTOR AND ITS INHIBITOR IN GUINEA-PIG SERUM.

A. A. MILES AND D. L. WILHELM

From the Lister Institute of Preventive Medicine, London, S.W.1.

Received for publication October 21, 1954.

THE reactions of capillary blood vessels in early stages of inflammation include vasodilatation, increased stickiness of the endothelial lining, increased permeability to soluble blood substances and changes facilitating the migration of leucocytes into the tissue spaces. The increase in capillary permeability is prominent among these vascular signs. Endogenous substances have been described as natural mediators of this effect; in the wheal and flare skin reactions of man, and in certain allergic reactions, histamine and Lewis's (1927) H-substance (see, *e.g.*, Ungar, 1953); and in acute inflammation, Menkin's (1940) leukotaxine, which is apparently a family of polypeptides (Duthie and Chain, 1939; Spector, 1951) formed by the breakdown of body proteins. Lewis's H substance is often presumed to be histamine.

Vascular reactions to histamine and leukotaxine are readily studied in the skin of "blued" guinea-pigs; *i.e.*, animals with the vital dye Pontamine Sky Blue in their circulation. Both substances increase capillary permeability to the dye, and thereby induce an intense blueing at the site of an intracutaneous injection. The capillaries recover their normal low permeability after a characteristic period of 10–20 min., and become resistant to further induction of blueing, by either histamine or leukotaxine, for several hours. Since blueing by leukotaxine, like blueing by histamine, is antagonised by mepyramine, it appears that leukotaxine acts by liberating endogenous histamine (Miles and Miles, 1952). However, the increased capillary permeability following injury is not always associated with a demonstrable liberation of histamine; and it may differ from histamine-induced permeability in its time course. Thus we have induced blueing in the guinea-pig skin by pinching, scratching, pressure; by the application of a turpentine poultice or mild blistering agents; by the intracutaneous injection of hypotonic saline, or of calcium chloride (0·02 M in 0·6 per cent saline); but in none of these instances did mepyramine maleate (neoantergan) antagonise the blueing effect. We found, moreover, that normal low permeability of the capillaries was not restored until after the lapse of two or more hours.

We obtained our first hint of an endogenous permeability-increasing substance other than histamine or leukotaxine while investigating cross-resistance to histamine-blueing. Nearly all substances which induce blueing, whether they are demonstrably histamine-liberators or not, will for a few hours render the site of intracutaneous injection resistant to further blueing agents, whereas physiological saline or Ringer's solution, which do not blue, do not induce resistance (Miles and Miles, 1952). Fresh guinea-pig serum, however, itself

with very little blueing activity, and without any modifying action on blueing by histamine injected simultaneously with it, induced a substantial histamine resistance after the lapse of an hour. This contradiction was to some extent resolved when we discovered that the serum contained the precursor of a powerful permeability-inducing substance, which could be simply unmasked by a 1/100 dilution of the serum in 0·85 per cent saline (Mackay, Miles, Schachter and Wilhelm, 1953).

Mammalian sera have been shown to contain substances which are toxic or otherwise pharmacologically active in animals of an alien species (*e.g.*, Brodie, 1900). Any estimate of the significance of the toxicity of such zoologically heterologous sera must, however, be dominated by considerations of species incompatibility; but the existence in homologous sera of the precursor of a strong permeability factor (PF), readily activable *in vitro* by simple dilution, and, as we later found, of an inhibitor of this factor (IPF), suggested to us that we might be dealing with endogenous substances of pathological if not physiological importance. In this paper we describe the conditions of the *in vitro* activation of PF and its relation to IPF.

MATERIALS AND METHODS.

Albino guinea-pigs, 450–650 g. body wt. were used. Some had been immunised with B.C.G. for previous tuberculin assay, but all results obtained with such animals were confirmed with normal guinea-pigs.

The skin of the trunk was depilated, after clipping away the hair, by a paste consisting of yellow barium sulphide 2 parts, and the detergent "Tide" 1 part, mixed to a smooth paste with 10 per cent glycerol in water 10 min. before use (Pitesky and Last, 1948). The depilated area was thoroughly washed with warm water. For permeability tests each animal received 65–75 mg. Pontamine Sky Blue 6 X (G. T. Gurr) per kg. body wt., given intravenously in a 5 per cent solution in 0·425 per cent saline. Animals so injected are referred to as "blued" and were used for tests without delay. The sites of pre-existing inflammation or of injury during depilation become bright blue within a few minutes of blueing; animals with such lesions were discarded.

Intracutaneous injections were given into the skin of the trunk in the region posterior to the shoulder blade and anterior to the knee joint in the sitting animal, and omitting the thin skin about 3–4 cm. on each side of the ventral midline. All solutions for injection were made up in 0·85 per cent saline, which by itself induces no blueing. The volume injected was 0·1 ml., which initially raises a bleb 9–11 mm. in diameter. With short-bevel no. 26 gauge needles, a small moderately pale area of traumatic blueing 1–4 mm. in diameter develops at the centre of the bleb. Consequently, it is only when the diameter of the area of blueing exceeds 3·5–4 mm. that the increase in capillary permeability induced by an injected substance can be considered significant.

The sites of the various intracutaneous injections in any one test were partly randomised and each injection was made in at least four guinea-pigs. Within the area used for these tests, the skin behaves uniformly to substances which increase capillary permeability. These intracutaneous injections were randomised not so much in the anticipation of heterogeneity, as to disguise the order of serial dilutions and so forth, and thus eliminate in subsequent measurements the unavoidable bias that occurs when the relation of lesions to the experimental design can be recognised from their position on the skin. Besides lesion diameter of the blue areas at the sites of injection, the intensity of colour of these lesions was recorded in a grade of ascending intensity: tr. (= trace), ±, ±, +, +±, ++, +++ and ++++. In general, the intensity of colour was roughly proportional to the lesion diameter, but the evaluation of the intensity of colour was very useful in demonstrating, *e.g.*, the efficacy of certain inhibitors, where lesion-colour, *i.e.*, intensity of exudation, was affected more than lesion diameter. In all the graphs of lesion diameter, each point is the mean of at least 4 lesions each in 1 animal and sometimes of as many as 16 lesions, 4 from each animal.

The duration of increased capillary permeability, the induction of resistance to permeability factors, and the avidity of tissues for the permeability factors, were measured by the methods of Miles and Miles (1952).

" Fresh " serum was collected by cutting the throats of stunned guinea-pigs, after the hair of the throat had been removed by close clipping. Unless otherwise stated, the blood was allowed to clot at room temperature for 5 min., the clot separated from the walls of the container, and the serum harvested after a further period of not more than 2 hr. at 4°. Throughout the year, " room temperature " ranged from 18–26°, but for most of the time the range was 19–22°.

The soya bean trypsin inhibitor (SBTI) and pancreatic trypsin inhibitor (PTI) were supplied by Worthington Biochemical Sales Co., New Jersey, U.S.A. The SBTI proved to be equal in potency to a sample of the highly purified material made by Dr. M. Kunitz, which we obtained through the courtesy of Dr. R. G. Macfarlane. The concentrations of substances in mixtures are given as final concentrations, unless otherwise stated.

Activation of a Permeability Factor (PF/Dil) by Dilution of Fresh Serum.

When 0·1 ml. volumes of serial dilutions of fresh guinea-pig serum in 0·85 per cent saline are injected intracutaneously into " blued " guinea-pigs, within 3 min. capillary permeability is substantially increased and the circulating dye exudes into the injection site to form a circular blue lesion. Fig. 1 summarises

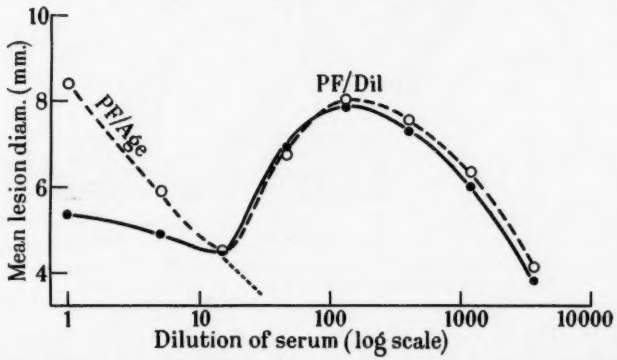


FIG. 1.—The activation of the permeability factor PF/Dil by dilution of fresh guinea-pig serum in 0·85 per cent saline, ●——●. A second permeability factor, PF/Age, which has matured in the same specimen of serum held at 2° for 6 days, superimposed on the PF/Dil activation curve, ○— —○.

In this and succeeding Figures serum dilutions are recorded as reciprocals.

the effect of dilutions that have stood for 1 hr. on the bench before injection. Maximum lesion diameter and maximum intensity of blueing occur in the 1/100–1/400 dilutions. Smaller lesions are produced by 1/1 to 1/15 serum, and the blueing is relatively feeble (±). Beyond 1/400, the blueing is still intense, but the size of the lesion declines with further dilution, and at about 1/5000 it is little more than that caused by needle trauma. This phenomenon was observed consistently with over 100 preparations, of either single sera or pools of sera. It cannot be attributed to any incompatibility between individual animals because it is readily elicited in guinea-pigs tested with their own serum, obtained from blood drawn 4 hr. previously. It was also observed in serum after storage for 17 days at 2° and for 82 days at — 10°. Serum was similarly activated by

dilution in phosphate buffer, pH 8, I = 0·2, and in mammalian Ringer's solution. Preparations of citrated plasma and heparinised plasma, like fresh serum, were activated by dilution with saline. This permeability factor we call PF/Dil, to distinguish it from a second permeability factor, PF/Age, which appears in serum on storage (see below).

The degree of activation in saline also depends on the temperature and age of the serum dilution. A few experiments indicated that maturation of PF/Dil in diluted serum was slow at 2° and about equally rapid at 20° and 37°. Its time course at room temperature is summarised in the three-dimensional graph

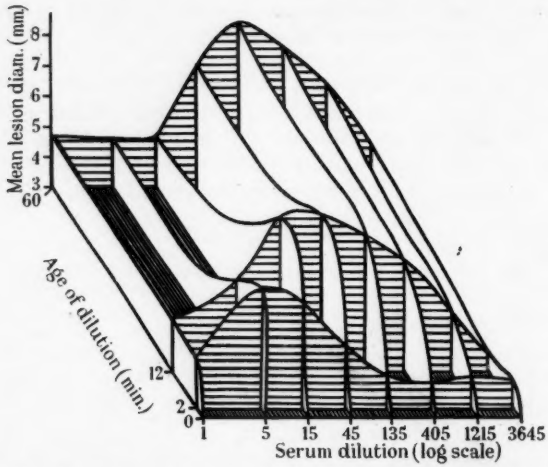


FIG. 2.—The progressive activation at room temperature of PF/Dil in serial saline dilutions of guinea-pig serum after intervals of 2, 12 and 60 min.

(Fig. 2), the curves of which were fitted by eye from the results of a titration of a fresh serum from a single guinea-pig, tested 2, 10–15 and 60 min. after dilution. The maximum activity occurs first in low dilution and shifts to a higher dilution of about 1/135 within the hour (*cf.* Fig. 1). Similar titrations revealed that beyond 1 hr. there was a continued but slower shift of the maximum; thus, in dilutions standing 1 day at 2°, the peak moves to about 1/1200, and after 4–7 days, to about 1/3600. The end-point also shifts to higher dilutions, though proportionately less than the peak (Fig. 3). We adopted activation after 1–1½ hr. at room temperature as a routine; and unless otherwise stated PF/Dil is the material so activated. It is evident from the time course of the 1/5, 1/15 and even the 1/45 dilutions, that the striking absence of a permeability factor in less dilute serum at 1 hr. represents the end result of a rapid initial activation and a slower subsequent inactivation, whereas in the more dilute serum, 1/135 and beyond, there is increasing activation during the whole period.

IPF, the inhibitor of PF/Dil.

The appearance of a peak in low dilution after 2 min. and its later shift to high dilution rules out any hypothesis of activation only in high dilution. We

may postulate, in addition to PF, a substance inhibiting it, symbolised by IPF, which in the lower dilution antagonises the activated PF. It would be simplest to assume that in undiluted serum the inactive form of PF was a complex of PF and IPF. This entails the additional assumption that after the rapid splitting of the hypothetical (PF + IPF) complex into free PF and IPF on dilution, a further change initiates a slow recombination of the split products. We should then expect to find inhibiting amounts of IPF wherever PF was manifested, including dilutions of 1/135 or greater; to explain the progressive uninhibited maturation of PF at, *e.g.*, 1/135 (Fig. 2), we must further assume a destruction of the newly liberated IPF in the highest dilutions.

The most economical hypothesis avoiding these additional assumptions is that the inactive precursor of PF/Dil in native serum is quite distinct from the

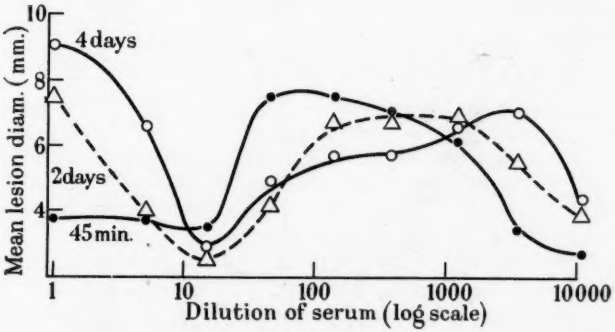


FIG. 3.—The progressive activation of PF/Dil in serial serum dilutions after an interval of 45 min. at room temperature, and subsequently after 2 and 4 days at 2°.

IPF; that there is in native serum a slowly-acting IPF, which is either not very potent or is present in small amounts, so that, at any rate after the lapse of 1 hr., it fails to inhibit all the PF which is activated in dilutions beyond 1/50. The maximum PF activity in dilutions of about 1/135, then, represents the end point of effective IPF inhibition after 1 hr.; after 24 hr. or more, part of the free PF present at 1 hr. has been inhibited by the small amount of slowly-acting IPF present, and the maximum PF activity moves into a more dilute reacting system, where, because of its progressive activation, more PF is present than there was after 1 hr.

The presence of free IPF in lower dilutions is evident from the inhibitory action of 1/2, 1/5 and 1/15 serum on a solution of activated PF/Dil. Four identical series of dilutions of a serum, over a range including the point of maximum activation, were activated by standing for 90 min.; an equal volume of saline was added to each dilution of the first series, and to those of the other three were added respectively equal volumes of the same serum, undiluted and freshly diluted 1/2·5 and 1/7·5. These mixtures were injected after a further 30 min. at room temperature. A detailed analysis of the resulting curves is difficult because of the complexity of the reacting systems in the mixtures, but, in general, it is clear from Fig. 4, which records those for 1/5 and 1/15 serum, that activated PF/Dil

is inhibited by moderately dilute sera, and that 1/15 serum has a greater IPF potency than 1/5 or 1/2 serum. (The curve for 1/2 serum, which is not recorded, was only slightly above that for 1/5 serum.) This suggests that some IPF is activated by dilution. However, the degree of dilution necessary for activation is less than that for PF/Dil. It can further be deduced that in 1/15 serum there is IPF in excess of the amount needed to keep in check any contained PF activated at that dilution. We must therefore conclude that the small activity of recently made low dilutions of serum (*cf.* the 2 min. curve in Fig. 2) is due not only to the action of relatively concentrated IPF, but also to a feebler activation of the PF-precursor in low dilutions. IPF acts relatively slowly; for maximum inhibition of PF/Dil, the mixtures with 1/5 serum must be held for at least 30 min. at room temperature before intracutaneous injection.

Properties of PF/Dil.

Heat-resistance.—The degree of heating which inactivates complement, 30 min. at 56°, does not affect PF/Dil. As Fig. 5 shows, the PF activity of high

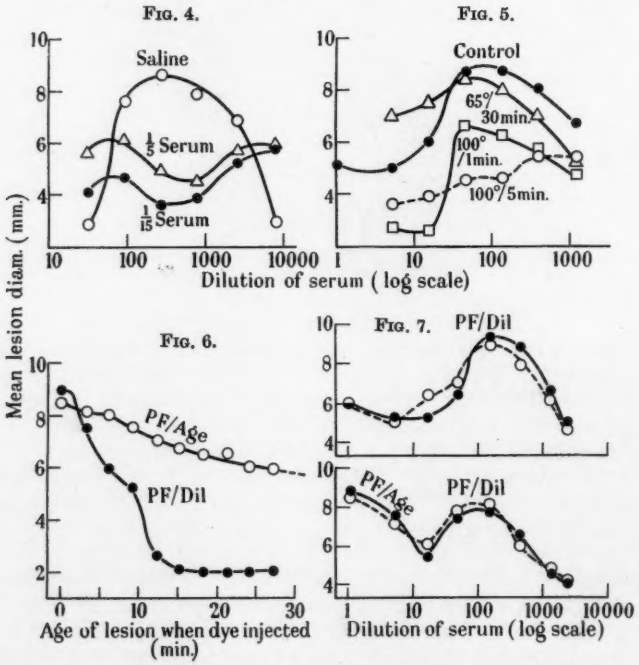


Fig. 4.—The inhibition of PF/Dil by an inhibitor, IPF, present in low dilutions of fresh serum.
Fig. 5.—The heat lability of PF/Dil. The effect of heating serial dilutions of serum in which PF/Dil has been activated.
Fig. 6.—The duration of increased permeability induced by PF/Dil and PF/Age (see text).
Fig. 7.—The inability of the antihistaminic drug mepyramine maleate to modify the permeability effect of PF/Dil and PF/Age. The broken lines connect readings from guinea-pigs given mepyramine, 20 mg./kg., 2 hr. before testing; solid lines connect readings from untreated controls.

dilutions of serum is almost completely destroyed after 5 min. at 100°, but heating does not destroy the activity proportionally in all dilutions. With progressively greater heating, the zone of inactivity in the less dilute serum widens; and the peak activity declines, but remains in about the same dilution. In the highest dilution, 1/1200, after an initial decline, the activity changes little, and indeed is the maximum activity in the dilution series heated for 5 min. at 100°. These results are consistent with the hypothesis that the disappearance of PF is due to its inhibition by IPF, whose slow action is accelerated by heating. In fact the curves in Fig. 5 bear a general resemblance to those in Fig. 3, where the gradual widening of the zone of inactive lower dilutions, with a consequent shift of fresh activity to the higher dilution, is interpreted as due to the slow inhibition of PF by the small quantities of IPF present. Whether this hypothesis is correct or not, in view of the fact that we are dealing, in all dilutions, with a mixture of serum proteins known in other circumstances to form inactive complexes on heating (see, *e.g.*, Bawden and Kleczkowski, 1942; Kleczkowski, 1945) it would be unwise to attribute to the hypothetical factors the heat lability they display as PF/Dil and as serum IPF.

Diffusibility.—PF/Dil is presumably a large-molecular substance, since there was no change in the titration curve of a series of serum dilutions after each had been dialysed in cellophan sacs for 24 hr. at 2° against 0·85 per cent saline.

Duration of increased permeability.— In the blued animal, the increased permeability was evident within 3 min. of the intracutaneous injection of a fully activated serum dilution and the lesion reached a maximum size and intensity within 20 min. The duration of permeability was measured by injecting into depilated guinea-pigs 0·1 ml. of 1/135 serum at 3-min. intervals, and giving dye intravenously immediately after the last intracutaneous injection. Thus, when the dye is injected after half an hour, the skin bears lesions varying in age from 0 to 30 min. and injection sites at which the vessels are no longer permeable do not blue. The permeability decreases steadily, and normal low permeability is restored after 15 min. (Fig. 6). The recovered capillaries become resistant to further applications of PF/Dil and of histamine; the resistance is maximal after 1 hr. and is disappearing after 5 hr.

Susceptibility to an antihistamine drug.—Histamine does not appear to be a mediator of the increased permeability. The dilution curves in guinea-pigs receiving 20 mg./kg. mepyramine maleate intraperitoneally 2 hr. before the test, do not differ significantly from those in control animals (Fig. 7). This dose of mepyramine is sufficient to diminish the permeability-increasing potency of powerful histamine-liberators by 3-fold or more (Miles and Miles, 1952).

Susceptibility to protease inhibitors.—The presence in serum of a biologically active, presumably large-molecular substance, activated by dilution, and of an inhibitor for that substance, is strikingly reminiscent of the plasma fibrinolysins and plasma fibrinolysin-inhibitors (Macfarlane and Pilling, 1946). A further general similarity was evident in the susceptibility of PF/Dil to soya bean trypsin inhibitor (*cf.* Kunitz, 1946–7). Equal volumes of solutions of the inhibitor (SBTI) and 1-hr.-old serum dilutions were held for 30 min. at room temperature, with an appropriate saline control series. The effect on the dilution curve is evident in Fig. 8, which shows the full inhibition of PF/Dil by a final concentration of 5 μg./ml. SBTI and partial inhibition by 2·5 μg./ml. Pancreatic trypsin inhibitor (PTI), on the other hand, in final concentrations of 20 μg./ml., only

slightly depressed the dilution curve ; 200 μg./ml. was more obviously inhibitory (Fig. 9), but its effect was only of the same order as that produced by 2·5 μg./ml. of SBTI. In contradistinction to fibrinolysin (Ungar, Damgaard and Hummel, 1952) PF/Dil was unaffected by prior incubation of the serum dilutions for 30 min. at 37° with 0·001 M and 0·01 M sodium salicylate.

Action on tissues.—Intracutaneous PF/Dil induced no obvious histological change, as observed in stained sections of skin excised 30 and 60 min. after the injection of 0·1 ml. of PF/Dil over the range 1/135 to 1/3645. The 60-min.

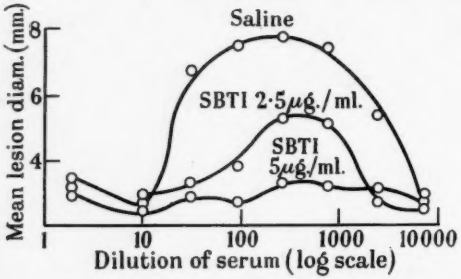


FIG. 8.—The inhibition of PF/Dil in serial dilutions of serum by soya bean trypsin inhibitor.

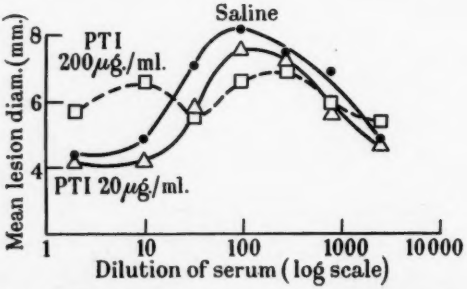


FIG. 9.—The inhibition of PF/Dil in serial dilutions of serum by pancreatic trypsin inhibitor.

lesions with undiluted serum and with 1/15 serum contained a small number, and with 1/5 serum a moderate number, of polymorphonuclear leucocytes in the perivascular tissues.

Maturation of a Permeability Factor (PF/Age) during the Ageing of Serum.

We have already noted that undiluted fresh serum (Fig. 1) sometimes induces a feeble blueing in the skin. This appears to be due to a second permeability factor which increases in amount and in blueing potency when the serum is allowed to age. This factor, which we designate PF/Age, is distinct from PF/Dil. In about 80 per cent of all our guinea-pigs, 0·1 ml. of sera less than 2 hr. old induced lesions 3–6 mm. in diameter, which were faintly or very faintly blue, with indistinct edges. In the remaining 20 per cent the lesions were faintly coloured, but 7–9 mm.

in diameter. Pooled serum behaved like single sera, but when animals were tested with their own serum the lesions were much smaller than those induced by the serum of other guinea-pigs. The frequency of the larger lesions increased when the serum was more than 4 hr. old, or when serum was removed from the clot within 30 min. of bleeding, instead of the routine 1–2 hr. The rate of maturation of this factor with age is evident in the Table. PF/Age also matures in

TABLE.—*The Maturation of PF/Age in Undiluted Guinea-pig Serum at 2°.*

Age of serum.	Approximate percentage of animals tested.	Lesion induced by 0·1 ml.	
		Size (mm.).	Intensity of colour.
2 hr.	80	3–6	tr to ±
	20	7–9	± to ±
4–24 hr.	50	3–6	± to +
	50	7–9	
2–3 days	100	8–10	+ to +±
6 days	100	8–11	+++

sera frozen at −10°, but takes many weeks to do so. Like PF/Dil, it does not dialyse through cellophan, is partly destroyed after 30 min. at 65°, and (Fig. 7) retains its potency in mepyramine-treated animals

The distinction of PF/Age from PF/Dil.

The relation of PF/Age to PF/Dil is displayed in Fig. 10, which records the titration of successive samples of a serum held at 2°, the dilutions of each sample

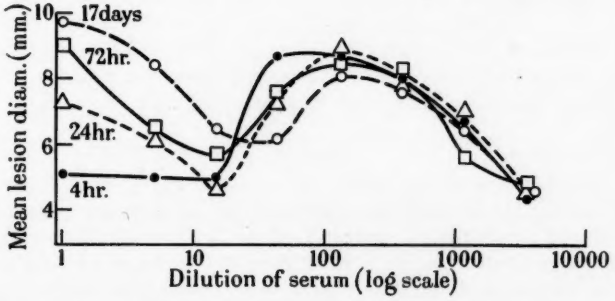


FIG. 10.—The maturation of PF/Age in serum held for varying periods at 2°. The serum samples were injected 90 min. after dilution, so that PF/Dil is also activated.

being held for 1 hr. at room temperature before injection. The PF/Dil maximum remains stationary. (This stationary maximum is not to be confused with the shifting PF/Dil maximum noted previously, which occurs when *serum dilutions*, not whole sera, are allowed to age.) PF/Age, on the other hand, not only increases in potency on ageing as indicated by an increase in the intensity of blueing, but in

amount ; because the end titre determined by extrapolations of the PF/Age curve moves from 50 to 200.

The end titres of PF/Age found in 48-hr.-old serum, and of PF/Dil in 1-hr.-old serum dilutions, provide a rough measure of the relative potency of the two factors. End-points, however, being extrapolations to the 4 mm. line of maximum traumatic blueing, are unsatisfactory responses for comparison. It is reasonable to assume that for both factors the rightward decline of the curves from peak activity represents the progressive dilution of active material. In all titrations, these curves were roughly straight and roughly parallel, with a slope of about −3·5 to −4·0 on the logarithmic scale of serum dilutions. It is therefore biometrically valid to compare doses at any desired response level. The 6 mm. lesion is a suitable response for this purpose, being near the mid-point of the decline between maximum diameter and 4 mm. ; we may conveniently define an effective blueing dose (EBD) as the amount in 0·1 ml. producing a 6 mm. lesion. On this basis, one EBD of PF/Age usually occurred at 1/8. In 1-hr.-old dilutions, one EBD of PF/Dil occurred at 1/2,000, and in one- or two-day-old dilutions at values up to 1/12,000. One ml. of serum therefore contains 80 EBD of activable PF/Age and from 20,000 to 120,000 EBD of activable PF/Dil ; so that PF/Age represents 0·4 per cent or less of the total permeability-increasing potency which can be elicited in guinea-pig serum.

Besides its characteristic dilution curve, its relatively low potency, and its slow activation in serum, PF/Age has other properties distinguishing it from PF/Dil. It induces exudation more slowly ; blueing is maximum in 60–90 min., compared with 20 min. for PF/Dil, and (Fig. 8) the increased permeability lasts more than 30 min. It is not inhibited on admixture with 1/2 to 1/15 dilutions of fresh serum (IPF) ; it is only slightly affected by high concentrations of SBTI (100 μg./ml.) and not at all by PTI (200 μg./ml.).

These facts, in our opinion, establish PF/Age as distinct from PF/Dil, and our subsequent investigations were confined to the factors activable in *fresh* serum and to those found in fractions of fresh serum.

The significance of our findings is discussed at the conclusion of our paper on the isolation and characterisation of the main permeability factor in fresh serum, and its inhibitor (Wilhelm, Miles and Mackay, 1954).

SUMMARY.

There is in fresh guinea-pig serum and plasma the precursor of a large-molecular substance which increases the permeability of guinea-pig blood capillaries. On the dilution of serum in 0·85 per cent saline, this permeability factor is rapidly and increasingly activated, substantial amounts being formed in dilutions of about 1/200 within 1 hr. at room temperature. It increases permeability as rapidly as histamine, leukotaxine and synthetic histamine-liberators, and, as with these substances, the capillaries recover their normal low permeability within 20 min. In low dilutions (up to 1/50) there is much less activation of the permeability factor and the material activated is antagonised within half an hour by a low-potency inhibitor, which appears to be present as such in serum, though more of it is activated on dilution to about 1/10.

The permeability factor is not antagonised by the anti-histamine drug mepyramine, or by sodium salicylate. It is antagonised moderately by pancreatic trypsin inhibitor and strongly by soya bean trypsin inhibitor.

When guinea-pig serum is allowed to age *in vitro* at 2°, a second permeability factor, distinct from the first, matures within a few days. Less than 0·5 per cent of the permeability-increasing potency activable in the serum can be attributed to this factor.

We are indebted to the Nuffield Trust for its financial help in the prosecution of this work; and to Dr. R. G. Macfarlane for a specimen of purified soya bean trypsin inhibitor.

REFERENCES.

BAWDEN, F. C. AND KLECZKOWSKI, A.—(1942) *Brit. J. exp. Path.*, **23**, 169, 178.
BRODIE, T. G.—(1900) *J. Physiol.*, **26**, 48.
DUTHIE, E. S. AND CHAIN, E.—(1939) *Brit. J. exp. Path.*, **20**, 417.
KLECZKOWSKI, A.—(1945) *Ibid.*, **26**, 33.
KUNITZ, M.—(1946–7) *J. gen. Physiol.*, **30**, 291.
LEWIS, T.—(1927) ' The Blood Vessels of the Human Skin and their Responses.' London (Shaw & Sons, Ltd.).
MACFARLANE, R. G. AND PILLING, J.—(1946) *Lancet*, ii, 562.
MACKAY, MARGARET E., MILES, A. A., SCHACHTER, M. AND WILHELM, D. L.—(1953) *Nature, Lond.*, **172**, 714.
MENKIN, V.—(1940) ' Dynamics of Inflammation.' New York (Macmillan).
MILES, A. A. AND MILES, E. M.—(1952) *J. Physiol.*, **118**, 228.
PITESKY, I. AND LAST, J. H.—(1948) *Science*, **108**, 657.
SPECTOR, W. G.—(1951) *J. Path. Bact.*, **63**, 93.
UNGAR, G.—(1953) *Int. Arch. Allergy*, **4**, 258.
Idem, DAMGAARD, E. AND HUMMEL, F. P.—(1952) *Amer. J. Physiol.*, **171**, 545.
WILHELM, D. L., MILES, A. A. AND MACKAY, MARGARET E.—(1955) *Brit. J. exp. Path.*, **36**, 82.

ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION.

II. ISOLATION AND PROPERTIES OF THE PERMEABILITY FACTOR AND ITS INHIBITOR.

D. L. WILHELM, A. A. MILES AND MARGARET E. MACKAY.*

From the Lister Institute of Preventive Medicine, London, S.W.1.

Received for publication October 21, 1954.

WE have described a permeability factor (PF/Dil), produced from fresh guinea-pig serum when diluted about 1/200 in 0·85 per cent saline, which is susceptible to soya bean trypsin inhibitor, and a low-potency inhibitor of this factor (IPF) apparently present as such in undiluted serum, but of which more is activated on dilution to 1/10 (Miles and Wilhelm, 1954). Like histamine and histamine-liberators, the PF on intracutaneous injection into the guinea-pig rapidly induces an increased permeability of the blood capillaries to a circulating blue vital dye. Using this permeability effect in guinea-pigs with Pontamine Blue in their circulation (" blued " animals) as our chief indicating reaction, we have prepared from guinea-pig serum fractions of the serum proteins containing respectively a high potency PF, and a relatively potent IPF, which appear to be identical with the corresponding factors in dilutions of serum ; and have determined some of their outstanding physico-chemical and biological properties.

MATERIALS AND METHODS.

The methods of measuring increased capillary permeability in the albino guinea-pig and of obtaining fresh serum are detailed in our previous paper (Miles and Wilhelm, 1954). For the accurate assay of PF potency, the injections, in 0·1 ml. volumes, of the various doses of preparations under test were randomised in a 4 × 4 Latin square, with two rows on each side of the midline, in 4 or more animals. In general, the diameter of the blue lesions, measured as a routine 30 min. after injection of the material under test, was linear with respect to log. dose and the slopes of the dosage-response lines were substantially parallel. It was therefore possible to estimate the relative potency of two preparations from the horizontal logarithmic distance between the two parallel response lines ; the antilog. of this value is the potency ratio (*cf.* Miles and Miles, 1952).

For fractionation of " fresh " serum, pools of clotted guinea-pig blood were allowed to stand overnight at 2°, and the harvested serum stored at − 10° until treated. No difference was observed between fractions made from sera treated immediately after harvesting and after storage for considerable periods at − 10°. Unless otherwise stated, fractionation was carried out by a modification of the method of Kekwick and Mackay (1954), using aqueous ethyl-ether systems, at controlled ionic strengths, pH and temperature. Some preparations were further fractionated in the preparative electrophoretic cell (Kekwick, Lyttleton, Brewer and Dreblow, 1951). Electrophoretic and ultracentrifugal analyses were made by the methods of Kekwick and Mackay (1954). The amounts of all serum fractions, unless otherwise stated, are cited as protein (6·25 × nitrogen content, estimated by the micro-Kjeldahl method).

Histamine was used as the acid phosphate ; weights are cited as that of the base. The preparation of leukotaxine was that used by Miles and Miles (1952). The histamine liberator 48/80 was supplied by Dr. E. J. de Beer of the Wellcome Research Laboratories, Tuckahoe, U.S.A. The soya bean trypsin inhibitor (SBTI) and pancreatic trypsin inhibitor (PTI) were

* Medical Research Council, External Staff.

supplied by Worthington Biochemical Sales Co., New Jersey, U.S.A. The concentrations of substances in mixtures are given as final concentrations, unless otherwise stated.

The hypotensive effect of various serum fractions was tested in the guinea-pig, rabbit, cat and dog. In the guinea-pig, arterial blood pressure was recorded by a small mercury manometer connected to the carotid artery of animals > 900 g. anaesthetised with chloralose (50 mg./kg.) and urethane (500 mg./kg.) intraperitoneally. Rabbits and dogs were anaesthetised with nembutal, 30 mg./kg., and cats with chloralose, 100 mg./kg. In all animals, anaesthesia was completed when necessary with ether. Intravenous injections in the guinea-pig were made through a blunted curved hypodermic needle tied into the external jugular vein, in volumes of 0·2–3 ml. In other animals injections were made *viâ* a glass cannula into the femoral vein. Respiratory changes were recorded by a tambour connected to a tracheal cannula.

For studying the release of histamine, isolated perfused preparations of cat and dog were prepared by the method of Feldberg and Paton (1951), modified according to Feldberg and Schachter (1952); and incubation of minced guinea-pig skin was carried out as described by Schild (1939).

Other methods of investigation are detailed in the relevant experimental sections.

RESULTS.

The Isolation of PF, the Permeability Factor in Serum.

PF in serum fractions.—Mackay (to be published) has applied to a number of mammalian sera a method of successive precipitation of serum proteins in aqueous ethyl-ether systems. Guinea-pig serum yields three main fractions: G2, chiefly α- and β-globulins; G3, chiefly γ-globulin; and AP, which is crude albumin; their relations to one another are shown in Fig. 1. The composition of the various fractions from a typical batch of pooled guinea-pig sera is set out in Table I.

TABLE I.—*Cholesterol Content and Electrophoretic Protein Components of Guinea-pig Serum and Serum Fractions.*

Material.	Cholesterol (mg./100 ml.).	Percentage protein content by electrophoresis.				
		Albumins.	Globulins.			
			α_1	α_2	β	γ
Serum	50·6	55	9	11	10	15
G2	346·0	—	—	46	54	—
G3	<5·0	10	—	8	17	65
G1S/P	<5·0	—	90	6	4	—
AP	<5·0	93	7	—	—	—

The serum was run in glycine buffer, pH 9·0, I = 0·2 (Clarke, 1928), the serum fractions in phosphate buffer, pH 8·0, I = 0·2.

In all guinea-pig sera so fractionated, the greater part of the PF activity was found in the G2 fraction. For example, in a comparative assay of fractions from a single pool, G2 was 24 times as potent as G3 and AP was inactive in concentrations up to 0·5 per cent (Fig. 2). Low activity was found in AP from some other batches, but in 10 fractions purified by removal of globulin contaminants the PF activity disappeared.

By electrophoretic fractionation, G2 was divided into relatively pure preparations of the α- and β-globulins (G2α and G2β). In 6 different batches of G2, PF moved consistently with the α-globulins. The G2α had between 2·4 and 3·3 times the potency of G2 (5 batches) and between 16 and 24 times the potency of G2β (4 batches). Fig. 3 records a typical assay.

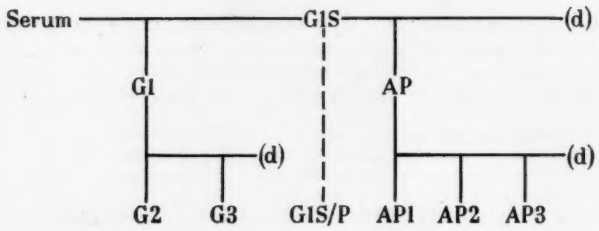


FIG. 1(*a*).—Scheme of the order of fractionation of guinea-pig serum. Horizontal lines indicate supernatant solutions; vertical lines indicate precipitates.

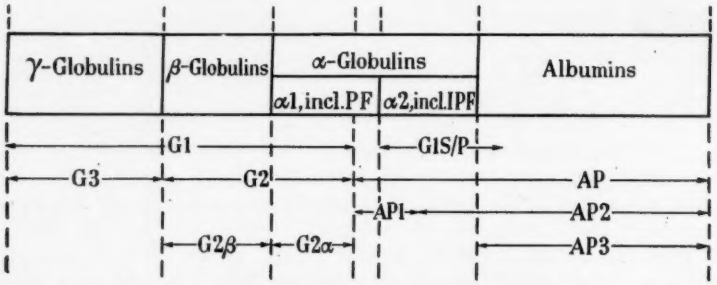


FIG. 1(*b*).—Scheme of the approximate relations between various fractions of guinea-pig serum, obtained by chemical and electrophoretic fractionation, in terms of the "spectrum" of the main electrophoretic components.

G1 = mixed globulins.
G2 = α- and β-globulins.
G3 = γ-globulin.
G1S = supernatant liquid from G1.
G1S/P = fraction rich in serum inhibitor (IPF).
AP = crude albumin contaminated with PF and IPF.
AP1 = fraction containing most of the G2 contaminants in AP.
AP2 = refined albumin plus IPF.
AP3 = homogeneous albumin only.
(*d*) = various discarded liquid remainders.

From the analysis of several batches, the approximate relative potencies of unit weight of the various fractions may with some confidence be summarised as follows:

Fraction.	Potency as PF.
G3(γ)	1
G2β	4
G2	24
(Histamine . . .	60)
G2α	72

In all respects the permeability factors in G2 and G2α behaved alike. We have therefore designated the PF in G2 as PF/G2α. The properties of PF/G2α described below are those of preparations of G2 as well as the purer G2α.

PF in plasma and blood cells.—The biological properties of G2, G3 and AP from a pool of 0·6 per cent citrated guinea-pig plasma were similar to those of the corresponding serum fractions. Since some of the serum pools contained traces of haemoglobin, we excluded the possibility that PF might be derived from

the cellular elements of the blood. The washed clot and red cells from a pool of blood after the separation of serum were haemolysed with an equal volume of distilled water. The resulting fluid, which contained 4·5 per cent protein, was fractionated by the routine method for serum ; the fraction corresponding to G2 contained no PF.

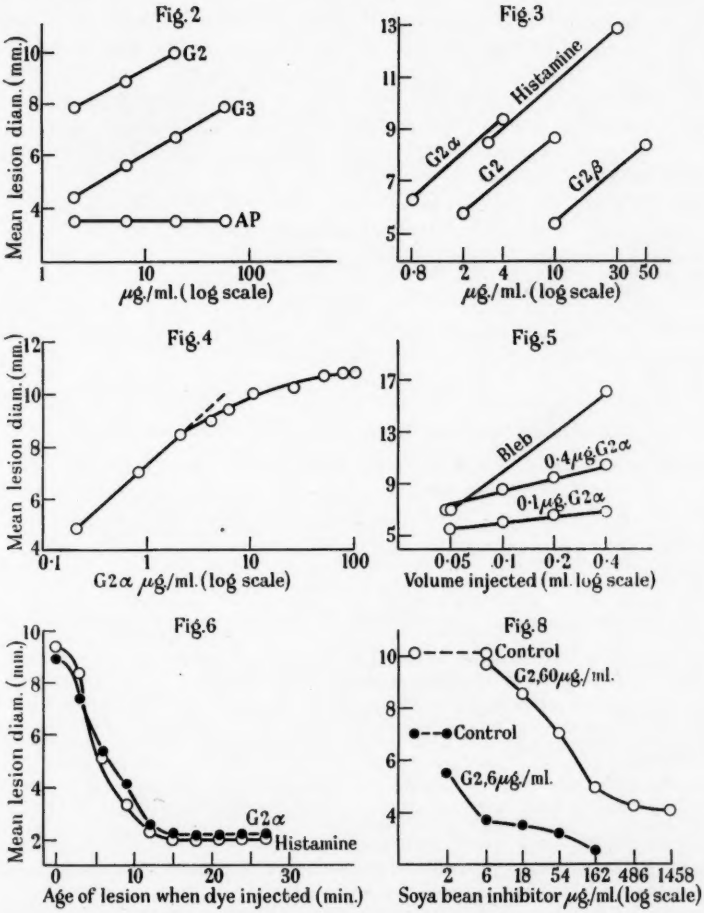


FIG. 2.—Relative permeability-increasing potency of G2, G3 and AP fractions of guinea-pig serum, by the intracutaneous technique in blued guinea-pigs.

FIG. 3.—Relative permeability-increasing potency of G2, of its electrophoretic components G2α and G2β, and of histamine, measured in blued guinea-pigs.

FIG 4.—Dosage-response curve for PF/G2α. Cutaneous lesion-diameter in blued animals is plotted against log. concentration in a constant injection volume.

FIG. 5.—Constant-dose intracutaneous titration of PF/G2α.

Fig. 6.—The duration of increased permeability induced by PF/G2α and histamine (see text).

FIG. 8.—The inhibition of PF/G2 by soya bean trypsin inhibitor.

Properties of PF/G2α.

G2 is least soluble at pH 5·0 at low ionic strength and low ether concentration. This fact, and a comparison of the mobility in the Tiselius apparatus of the proteins of G2 with those of whole serum, indicate that the fraction is a mixture of α_2- and β-globulins. The α_2 fraction is collected as the leading component of the two in the preparative electrophoresis apparatus. The fraction G2 contains the bulk of the serum lipid, assayed as cholesterol (Table I), removable by ether (McFarlane, 1942) and by ether-alcohol extraction (Hardy and Gardiner, 1910), and may consequently be defined as a mixture of α- and β-globulins, including lipoprotein globulins. The PF is consistently associated with α_2-globulin. The PF activity of preparations of G2 is not destroyed when lipid is removed by extraction with ether.

Stability to heat and pH.—In concentrations of 15–2000 μg./ml. in saline, PF/G2α loses about 10–20 per cent of its potency when held at 98–100° for 2 min., 65–75 per cent after 5 min., and 80–90 per cent after 10 min. at 100° . There is no loss of potency in preparations of G2α dialysed for 48 hr. at 2° against acetate-phosphate buffers pH 4, 6 and 9, and subsequently for a further 48 hr. against pH 8 phosphate buffer. The pH 9 buffer was prepared by adding 0·2 M-NaOH to acetate-phosphate buffer.

Reactions to Local Injection of PF Preparations.

The dosage-response to intracutaneous PF.—In the blued animal G2α rapidly induces a round area of intense blueing within 2–3 min. of injection. The diameter of the lesion produced by low concentrations is linear with respect to the log. concentration, with a slope usually of 3·5–4·0 ; when lesion diameters of > 9 mm. are reached, the curve flattens and with high concentrations becomes almost horizontal (Fig. 4). Lesions induced by high concentrations of G2α have an uneven margin, with small " pseudopodial " prolongations of intense blueing.

The flattened slope of the response to high dosage is consistent with the assumption that the PF has a high tissue affinity. The outward radial spread of intracutaneously injected fluids takes place in two stages ; a rapid spread during injection, to form a bleb of 5 mm. radius, and a slow spread during which the diameter increases linearly with log. time (Miles and Miles, 1952). The proportional distance to which a contained substance spreads depends on the rate and firmness of its adsorption to the tissues. An avidly adsorbed substance, though it might travel with the saline a relatively great distance from the needle point during injection, when the radial 5 mm. spread of saline is rapid, would spread little further during the subsequent slower outward spread of the injection fluid. When the substance is in concentrations equal to or lower than that sufficient to reach the 5 mm. limit during injection, the radial spread would be proportional to its concentration ; in higher concentrations it would fill the 10 mm. initial bleb, but would spread little further, and the slope would change abruptly, as with PF in Fig. 4. This rapid fixation to the tissues is more precisely demonstrable in titrations of fixed amounts of G2 in varying volumes of saline ; the lower the affinity of a substance for skin tissue, the more nearly will its spread (lesion diameter) be directly proportional to the spread of the injection fluid (initial bleb diameter) ; and the higher the affinity, the more nearly will the final lesion diameter be independent of the injection volume. In Fig. 5 the slope of the lesion diameter for 0·4 μg. of G2 in graded injection volumes is 3·2 and for 0·1 μg. of G2 is nearly flat (1·4), indicating a high affinity, in striking contrast with the slope (10·0) for diameters of the blebs formed by the injection fluid. Measured in this way, histamine has a low, and leukotaxine a moderate, affinity for skin tissue (Miles and Miles, 1952).

Presumably it is the high tissue affinity of PF/G2α that limits the linear regression of diameter on log. dose to low concentrations of the factor. As a consequence, only responses less than 10 mm. in diameter, and greater than the 4 mm. maximum diameter of traumatic blueing usually encountered, are available for the accurate estimation of potency (*cf.* Fig. 3 and 4).

Potency of PF/G2α in terms of histamine.—As Fig. 4 shows, as little as 0·02 μg. G2α in 0·1 ml. saline is sufficient to increase permeability to Pontamine Blue. In this respect G2α has the same order of potency as histamine. A valid assay is possible because the slope of the dosage-response lines to the two substances is approximately the same (Fig. 3). In terms of histamine, the potencies of six different batches of G2α ranged between 0·80 to 1·68, with a mean of 1·2. PF/G2α is clearly a powerful inducer of capillary permeability.

All our evidence indicates that PF/G2α is an α-globulin. If we assume that the proteins of G2α have a molecular weight of 200,000, and accept its potency on a weight basis as 1·2 times that of histamine, then on a molar basis G2α is (1·2 $\times$ 200,000/111) approx. 2000 times as active as histamine. Since preparations of G2α certainly contain proteins other than the PF (see below), clearly the activity of the PF/G2α molecule is several multiples of 2000 times that of the histamine molecule. The molecular weight of 200,000 for α-globulins is an approximate mean of weights ranging from 78,000 to 300,000, estimated by Oncley, Scatchard and Brown (1947). Even if G2α proved to be a globulin of the lowest estimated weight, 78,000, the molar ratio of potency is still of the order of 850. Since histamine is one of the most powerful permeability factors known, the much higher activity of PF/G2α strongly suggests that its action is either enzymic or enzyme-inhibiting.

Duration of increased capillary permeability, and induction of resistance to permeability factor.—The increased permeability induced by 2 μg./ml. of G2α, like that of PF/Dil. and histamine, is maximal after about 3 min., and then declines steadily ; normal low permeability is restored within 12–15 min. (Fig. 6). Tested by the superinjection technique of Miles and Miles (1952), the recovered capillaries were found to have become resistant to further application of PF/G2α and of histamine ; the resistance was maximal at 1 hr. and disappearing at 5 hr.

Effect on diapedesis of leucocytes.—Two consequences of inflammatory change in blood capillaries—the adherence of circulating white cells to the endothelium and their emigration into the perivascular tissues—were investigated with respect to the serum fractions.

Intracutaneous injections of graded doses in 0·1 ml. saline of the various fractions and of histamine and leukotaxine were made into normal guinea-pigs, and the injection sites excised for histological examination after 30 and 60 min. Each dose was tested in two skin sites. Both the degree of tissue leucocytosis at the centre of the lesion and the extent to which intravascular leucocytes appeared to be adhering to the capillary endothelium and passing through it were compared with those in the normal skin at the periphery of the lesions and in the sites of injection of 0·85 per cent saline. Little change was visible after 30 min., except that the leukotaxine, in concentrations of 50–500 μg./ml., had induced some leucocytosis, the most effective concentrations being 400–500 μg./ml. Table II records only the results in the 60 min. lesions.

In these, only G2α, leukotaxine and histamine consistently induced a substantial tissue leucocytosis ; G2β, G3 and AP2 were inactive. Adherence of

TABLE II.—*Tissue Leucocytosis in the Guinea-pig 60 Minutes after Intracutaneous Injection of Various Guinea-pig Serum Fractions.*

Concentration (μg./ml.).	Substance injected.					
	G2α	G2β	G3	AP2	Leukotaxine.	Histamine.
50	+	+	±	±	0	+
100	++ (*a*)	0	tr	0	±	±
200	± (*a*)	tr	0	0	±	+
300	+	tr	tr	0	+	±
400	+++ (A)	0	0	tr	++ (*a*)	+
500	++ (A)	+ (*a*)	0	0	++ (*a*)	++

Designations.—G2α = α-globulin. G2β = β-globulin. G3 = γ-globulin. AP2 = refined albumin.

tr (trace), ±, +, ++, +++ = intensity of leucocytosis; +++ = infiltration of panniculus adiposus and dense perivascular collections of leucocytes in panniculus adiposus and deep dermis. (*a*), (A) = intensity of adherence of leucocytes to capillary endothelium and of diapedesis.

leucocytes to the endothelium and diapedesis of leucocytes were not constantly seen with G2α and less so with leukotaxine. The doses of G2α inducing both these effects are greatly in excess of those required to increase capillary permeability (*cf.* Fig. 4).

Effect on stickiness of capillary endothelium.—The adherence of leucocytes is usually attributed, in part at least, to an increased stickiness of capillary endothelium. Stickiness was demonstrated by the intravenous injection of 200 mg./kg. of a 5 per cent solution of saccharated iron oxide (see Cappell, 1929) immediately after the intracutaneous injection of G2α (0·5 μg.), histamine (1·0 μg.) and leukotaxine (7·5 μg.), each in 0·1 ml. saline. The suspension of saccharated iron oxide used (Hopkin and Williams) was stable in guinea-pig serum. The injection sites were excised from animals killed after intervals of 30 sec., 10, 30, 60 and 360 min., and iron was demonstrated in paraffin sections by Gomori's (1936) method.

Induction of stickiness was estimated in 60 min. sections by the amount of iron in the capillaries of the upper dermis on the one hand, and of the deep dermis, panniculus adiposus, panniculus muscularis and subcutaneous tissues on the other. Compared with injection sites receiving saline, the amounts of iron per unit area of tissue were as follows:

Injection of—	Upper skin.	Deep skin.
Saline	+	trace
Leukotaxine (7·5 μg.)	+++	+
Histamine (1·0 μg.)	++++	+
G2α (0·5 μg.)	++++	±

Compared with histamine and leukotaxine, the area affected by the G2α extended less far from the point of injection, with less involvement of the deep tissues, again emphasising the high tissue affinity of the G2α.

The accumulation of circulating iron oxide in the capillaries is visible in the living depilated animal as an area of pale brown staining, developing within a few minutes at the intracutaneous injection site. As with induced permeability to Pontamine Blue (*cf.* Fig. 6), lesions over 10 min. old stained only round the needle puncture. The degree of staining, and its dependence on *intra*-capillary iron oxide, was confirmed histologically. The amount of iron held was maximal in lesions 0 to 5 min. old; little was held by lesions 10–15 min. old, and lesions more than 20 min. old held no more than untreated tissues.

Although induced stickiness passes off after about 10 min., substantial amounts of the iron oxide which has adhered to the endothelium during this period remain adherent for up to 6 hr. (Wilhelm and Miles, to be published).

Reactions to systemic injection of PF preparations.

The intravenous injection of G2, of which approximately half is G2α, in doses of 5–10 mg./kg. body wt., produces a striking non-fatal shock. Within 30–60 sec., the cutaneous vessels flush, most noticeably in the ear ; respiration becomes rapid and heaving, and some animals make jactitating movements. These events are quickly followed by cyanosis, paralysis of the hind limbs and limp collapse, which last about 10 min. The animals are unsteady on their feet from the 10th to 15th min. After 15 min. they recover enough to stand again ; they are quiet for a further 15 min. and behave quite normally after 1 hr.

Large doses, of 15–20 mg./kg., induce rapid shallow respiration and marked opisthotonos followed by paralysis of the hind limbs and general collapse. The period of collapse is not longer with the larger doses, but recovery may take 40 min. Animals given small doses, 2·5–5·0 mg./kg., have respiratory embarrassment, cutaneous flushing and mild collapse, but recover completely in 5 min.

The intravenous injection of γ-globulin (G3) and of purified albumin (AP3) in doses of 10–20 mg./kg. caused only transient cutaneous flushing.

When animals with circulating Pontamine Blue are used, within 30 sec. of the intravenous G2, the eyelids become faintly blue ; at 45–60 sec. they are distinctly blue, and there is faint blueing around the nostrils, at the bases of the ears and the adjacent scalp and around the nipples ; within 80–90 sec. the blueing is generalised, but rather deeper in the areas first affected and at the back of the neck and over the lower end of the spine. Up to the third minute there is a steady increase in colour ; at 8–12 min., when it is maximal, the most feebly blued areas are in the central belly wall, the flanks, the lower thoracic area dorsally and the extremities of the limbs. The results resemble those produced by the histamine-liberator 48/80 (Feldberg and Miles, 1953), except that the blueing is much less intense and the distinction between lightly and more heavily stained areas much less obvious. The effect is clearly one of a general moderate increase in capillary permeability over the whole skin.

Effect on blood pressure : the hypotensive factor, HF.

Guinea-pig.—G2 was injected intravenously into 18 anaesthetised guinea-pigs, in amounts ranging from 25 μg./kg. to 1·5 mg./kg. All animals responded with an immediate fall of blood pressure, the most sensitive animals responding to 25 μg./kg. The degree and duration of the depressor effect varied directly with the amount injected, but with the above amounts, the arterial blood pressure always returned to its initial level within 6 min. or less (Fig. 7(a)). Hypotension was frequently accompanied by an increase in the rate and depth of respiration. The hypotensive factor (HF) was confined to G2α preparations ; albumin (AP), G2β and G3 fractions were without effect on intravenous injection in doses/kg. 10 times that of the effective doses of G2.

In two experiments, in which injections were given at 6 min. intervals, there was no significant decrease of the depressor response to a constant dose either of histamine (2 μg./kg.) or of G2 (100 μg./kg.), during 10 alternating injections of each substance. Diminished responses to the same dose of G2 were observed, however, when injections were repeated immediately or shortly after each recovery of the blood pressure, but the lesser responsiveness could be overcome by increasing the dose.

The relation between dose and degree of hypotension illustrated in Fig. 7(*a*) made possible a rough assay of the potency of various serum fractions. As with PF, the order of hypotensive potency was G2α > G2 > G2β, the last being inactive in the concentrations tested. Several comparisons were made of the PF and HF potency of G2 preparations, and in all, within the limits of error of the methods employed, the relative potencies were the same. Thus the PF potencies of 5 different batches were 0·8, 1, 1, 1, 0·2, and the corresponding HF potencies were approximately 1–2, 1–2, 1–2, 1 and 0·5. Moreover, on heating two batches to 98° for 10 min., the two potencies declined in parallel, indicating a similar thermolability. With one batch the PF potency declined from 1 to 0·2, the HF from 1 to 0·25 ; with the other, the PF from 1 to 0·7 and the HF from 1 to 0·75.

Dog.—G2 in doses of 200–800 μg./kg. produced in 5 anaesthetised dogs a marked immediate fall in the arterial blood pressure, lasting from 30 sec. to 3 min., usually accompanied by a slight to moderate increase in the rate and depth of respiration. The activity of G2 was concentrated electrophoretically in the α-globulin fraction, and albumin, β- and γ-globulin fractions possessed little or no activity (Fig. 7(*b*)). The HF potency in the dog, as in the guinea-pig, was only partly destroyed by exposure to 98° for 2 min.

Rabbit.—In 5 anaesthetised rabbits, G2, 0·4–2·0 mg./kg., produced an immediate fall of the arterial blood pressure lasting from 20 sec. to 1 min. This effect differed in two important respects from that caused by guinea-pig G2 in the guinea-pig and dog. The HF responsible was more heat-labile, being in every instance completely destroyed by heating to 98° for 1 min. ; on dividing G2 electrophoretically, the activity moved with the β, not the α fraction.

Cat.—Intravenous G2, in doses of 0·5–3·0 mg./kg. into 5 anaesthetised cats caused an immediate fall of blood pressure with recovery in 1–2 min. The albumin fraction AP2, 2·0 mg./kg., had no effect. The cat, unlike the guinea-pig, dog or rabbit, was susceptible to guinea-pig serum (Fig. 7(*c*)). The response to guinea-pig serum was in fact like that to horse serum, suggesting that both sera were inducing the hypotensive response described by Brodie (1900). But guinea-pig G2 itself did not produce the reflex cardiac slowing characteristic of the Brodie effect with serum and of intravenous injection of serotonin in the cat (Schneider and Yonkman, 1953). The HF effect was not abolished by section of the cervical vagi.

In all the animals the HF effect was found predominantly in the G2 fraction ; there was none in the albumin or γ-globulin preparations. In all but the rabbit it was present in the α-, not the β-globulin fraction. HF paralleled the PF in potency, and had the physico-chemical properties we have described for PF/G2α. The HF effect was unlike that of histamine, and in the guinea-pig and cat was unaffected by intravenous mepyramine maleate, 1 mg./kg., which completely abolished the response to histamine. Unlike the response to acetyl choline it was unaffected by atropine. It lacked the element of cardiac slowing in the cat, and the marked respiratory gasp produced by serotonin (5-hydroxytryptamine) in other species. However, it should be noted that other substances, *e.g.*, bradykinin, various peptides, adenylic acid and adenosine derivatives, also can produce hypotensive effects, without the distinguishing features of the serotonin and the Brodie effects.

The response of the dog to G2 resembles that of the guinea-pig. We discuss below the distinction of the effect and the Brodie phenomenon in the cat, and on p. 101 the implication of finding that the β-, not the α-globulins of guinea-pig serum are the more powerfully hypotensive in the rabbit.

Effect on circulating leucocytes.

G2 is not a leucocytosis-promoting factor (*cf.* Menkin, 1946). On 2 successive days, hourly total and differential counts were made of the white blood cells of

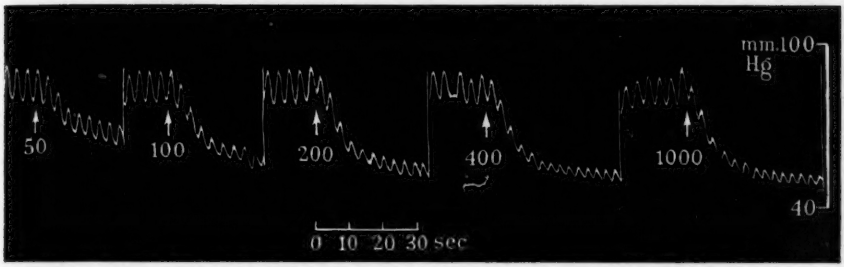


FIG. 7(*a*).—Hypotensive effect of intravenous G2 preparations in a 930 g. guinea-pig. The figures indicate μg./kg. G2 injected. The kymograph was stopped for 4 min., between each injection. For Fig. 7(*a*)–(*c*) horizontal scale = 10 sec. intervals; vertical scale = mm. Hg. arterial blood pressure.

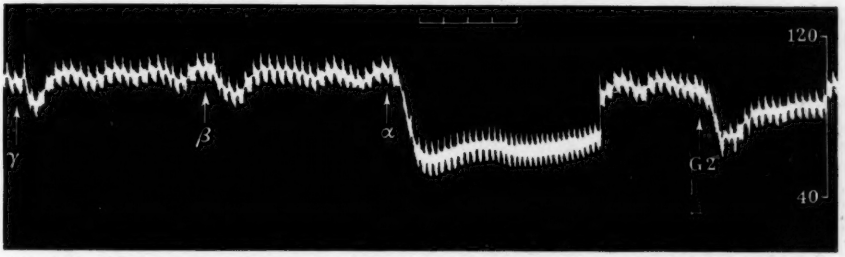


FIG. 7(*b*).—Hypotensive effect, in an 8 kg. dog, of various guinea-pig globulin fractions prepared from the same pool of serum.

γ = G3, 1 mg./kg.
β = G2β, 500 μg./kg.
α = G2α, 500 μg./kg.
G2 = G2, 1 mg./kg.

The kymograph was stopped for 4 min. at the vertical line after α.

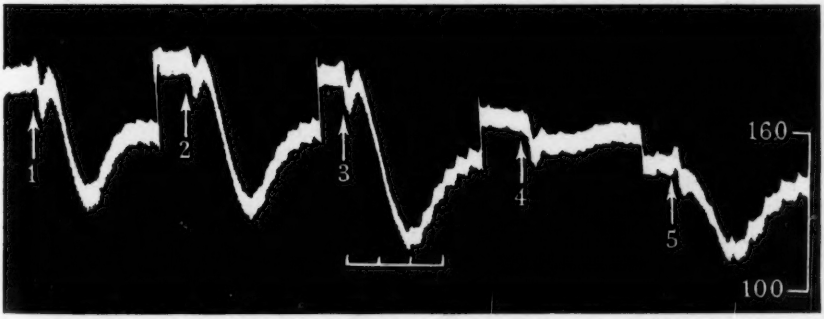


FIG. 7(*c*).—Hypotensive effect of various sera and serum fractions in a 2·6 kg. cat.

1 = guinea-pig serum, 0·5 ml.
2 = horse serum, 0·7 ml.
3 = horse serum, 0·7 ml., plus SBTI, 300 μg. (0·3 ml.) held at room temperature for 5 min. before injection.
4 = guinea-pig G2, 1 mg./kg. (2·6 ml.) plus SBTI, 300 μg. (0·3 ml.) held at room temperature for 5 min. before injection.
5 = guinea-pig G2, 1 mg./kg. (2·6 ml.).

12 guinea-pigs over a period of 7 hr. On the 2nd day, 6 received 1·0–2·5 mg./kg. intravenous G2, and 6 the same weight of the guinea-pig albumin fraction AP3.

A substantial rise in the first hour occurred in one G2-treated animal. Otherwise neither the G2 nor the AP3 induced any significant change in the circulating white blood cells.

The Mode of Action of PF and HF.

Exclusion of histamine-liberator effect.—Preparations of G2α, rich in PF and HF, do not cause contraction of the guinea-pig intestinal segment in Tyrode's solution in the organ bath. Nor do they liberate histamine from preparations of mammalian skin.

After the intra-arterial injection of 2 mg. G2 into two isolated perfused cat skin preparations, no trace of histamine was detectable in the perfusion fluid; negative results were likewise obtained on a similar preparation of dog skin. G2 was also incubated with minced guinea-pig skin (1 mg. to 1000 mg. of skin) according to Schild's (1939) method; and here again no histamine was liberated. All test preparations, however, readily released histamine on contact with known liberators such as 48/80 (Paton 1951) or egg white (Schachter and Talesnik, 1952). We described (Mackay, Miles, Schachter and Wilhelm, 1953) an antihistamine factor (AHF) both in diluted serum and in G2α, which both diminishes and delays the muscle-contractor action of histamine on the isolated guinea-pig intestine. G2 is not however a very potent AHF, and according to titrations in the organ bath, could have accounted for the suppression only of amounts that were very small in comparison with those liberated by subsequent treatment with known histamine liberators.

Effect of an antihistamine drug.—The absence of any direct evidence of histamine liberation was consistent with the absence of any inhibitory action by mepyramine maleate on either PF or HF.

Guinea-pigs received intraperitoneal injections of mepyramine 20 mg./kg., 2 hr. before the injection of G2. This dose is in great excess of that which totally inhibits the increased permeability effect of histamine in the skin, and significantly diminishes the lesion produced by histamine-liberators like leukotaxine and 48/80 (Miles and Miles, 1952). Mepyramine had no detectable effect on PF/G2α. Nor did it protect the animal from the permeability effect of intravenous G2α. Twenty guinea-pigs, of which 10 had received intraperitoneal mepyramine maleate, were given Pontamine Blue into one vein, and immediately after, 2·5 mg./kg. G2 into a second; after 40 min. the animals were killed and depilated. The 2 groups did not differ significantly in the degree of shock, or in the degree of blueing of skin and internal organs, except that the ears of the mepyramine-treated animals were less blue. The shock and blueing induced in similar conditions by the histamine-liberator 48/80 are almost entirely suppressed by mepyramine (Feldberg and Miles, 1953). We have already noted that mepyramine maleate does not antagonise the hypotensive effect of G2 in the guinea-pig or the cat.

There is thus good evidence that neither PF nor HF acts by the liberation of histamine.

Complementary and anti-complementary activity.—The fractions AP, G2α and G2β from Batch 16 (*cf.* Table IV) were examined for complementary activity by Dr. I. H. Lepow. G2α and G2β were anti-complementary, G2α being about $\frac{1}{4}$ as active as G2β in this respect; AP was not anti-complementary. None of the

fractions contained haemolytic complement. AP had 1/64th the C'2 activity of a specimen of fresh guinea-pig serum, and no C'1, C'3 or C'4. No components were detectable in the two G2 fractions, even in concentrations lower than those with anti-complementary activity.

Tests for enzymic activity.—We have as yet made only a limited and superficial investigation of substrates of the hypothetically enzymic PF/G2α, with uniformly negative results.

G2α contains no collagenase, tested by the azocoll method devised by Oakley, Warrack and van Heyningen (1946); no mucinase, tested with 1 per cent solutions of A and B blood group substances, which were observed for decrease of viscosity on incubation with G2 at 37°; no lecithinase, tested with egg yolk lecithin, with added calcium, to a concentration of 0·02–0·6 per cent, and against guinea-pig erythrocytes for lysis; no gelatinase, tested with 5 per cent solutions of gelatin preparations, also observed for decrease of viscosity; and no proteinase, tested with 0·2 per cent solutions of casein, which were observed for the liberation of tyrosine. These negative results do not exclude these various enzymes as causes of the *in vivo* PF and HF effects; the tests were not sufficiently extensive. Moreover, in making these *in vitro* tests, we may not have included co-factors whose presence in the tissues is necessary for the permeability effect.

The effect of enzyme inhibitors.—With two exceptions, our attempts to prove the enzymic nature of PF by the use of enzyme inhibitors were unsuccessful. Preparations of G2 were mixed for 30 min. at 37° with 0·001 M of the following substances, and when necessary, the mixtures neutralised with 0·1 N-NaOH: NaF, NaH_2PO_4, $Na_4P_2O_7$, $MnCl_2$, sulphosalicylic acid, sodium salicylate, iodo-acetic acid and menaphthone. Sodium salicylate was tested also at 0·01 M. None of them affected the permeability factor. The test is inconclusive, because some of the substances tested themselves increased capillary permeability in 0·01 M concentrations; and others, like $FeCl_3$, $HgCl_2$ and merthiolate, did so even at 0·001 M and could not therefore be used. Moreover, it is possible that an *in vitro* inhibition would be reversed when the mixture came into contact with the tissues on test injection. We also tested 19 common amino-acids representing those found, *e.g.*, in serum proteins. Here again the results were negative. This is not an unexpected result, because the permeability-inducing activity of the solutions of most of these amino-acids necessitated our using them in concentrations of 0·066 M in 0·85 per cent saline, and some of the less soluble acids in even lower concentrations; that is, in concentrations much lower than those shown to affect proteinases (*cf.* Grob, 1949–50).

Trypsin inhibitors.—As we have previously noted (Miles and Wilhelm, 1954) the similarity of the activation by dilution of a major PF in fresh serum and of serum fibrinolysin (Macfarlane and Pilling, 1946) led us to test the trypsin inhibitors from (*a*) soya bean (SBTI) and (*b*) pancreas (PTI).

(*a*) Graded concentrations of SBTI in saline were added to two concentrations of G2, and the mixtures tested intracutaneously after 30 min. at 37° and then 30 min. on the bench. As Fig. 8 shows, 6 μg./ml. of G2 preparation were completely inactivated by 6 μg./ml. of inhibitor and 60 μg./ml. of G2 by about 200 μg./ml. of inhibitor. The period of *in vitro* inactivation and the temperature of the mixture before injection are not critical; equally good inhibition occurs in 2 min. at room temperature and in 1 hr. at 37°.

It is noteworthy that the curves of neutralisation of equipotent amounts of G2α, G2 and G2β, by the SBTI added in graded doses of 0·28 μg. to 200 μg., proved to be substantially identical, indicating that these three preparations owe their blueing potency to the same kind of substance. We may also note in this context that SBTI in concentrations up to 486 μg./ml. does not alter the permeability effects of histamine, 5 and 50 μg./ml.; of the histamine liberator, 48/80, 5 and 50 μg./ml.; or of leukotaxine, 100 μg./ml.

The hypotensive factor also was antagonised to the same extent by SBTI. In two tests with the guinea-pig, an equal concentration of SBTI greatly decreased the HF effect of G2, 100 μg./kg. In the cat SBTI also inhibited the action of G2, and that of whole guinea-pig serum. The inhibitor also made possible a distinction between the HF and the Brodie effect, because the Brodie effect was unchanged when horse serum was incubated with SBTI, 300 μg. to 0·7 ml. serum, for 5 min. before injection (Fig. 7(*c*)).

The hypotensive effect produced in the rabbit by G2β was very susceptible to SBTI, which inhibited 10 times its weight of G2.

(*b*) Incubation for 30 min. at 37° with PTI in final concentrations up to 1458 μg./ml. had no effect on the PF potency of 60 μg./ml. G2, but 486 and 1458 μg./ml. partly inhibited that of 6 μg./ml. G2.

Fibrinogenolytic activity.

Since both PF and fibrinolysins are susceptible to trypsin inhibitors, tests were necessary to distinguish the two, or to prove their identity. The enzyme was assayed by measuring the rate at which a 0·2 per cent solution of fibrinogen in veronal buffer pH 7·2, became unclottable when incubated at 37° with a known amount of the fraction to be tested (Kekwick, Mackay, Nance and Record, to be published). This system was preferred to that of measuring the time of lysis of a fibrin clot (Macfarlane and Biggs, 1948) because thrombin preparations contain variable amounts of fibrinolysins. With both bovine and human fibrinolysins, fibrinogenolysis and fibrinolysis proceed at similar rates and measurement of the first activity is a reasonable indication of the second (Ratnoff, 1953). The end point is the time taken for the disappearance of the fibrinogen, as indicated by the absence of a fibrin clot 3 min. after the addition of thrombin to periodic samples of the reaction mixture. The relative content of fibrinogenolysins in the various fractions in any one comparative assay is indicated by the reciprocal of the "fibrinogen life", which, within the span of the life of the untreated fibrinogen, is inversely proportional to enzyme concentration. Comparison of different assays was made possible by using for all the tests a single batch of human fibrinogen having a life at 37° of 500 hr.

The fractions G2, G2α and G2β all contained fibrinogenolysins. This is to be expected, since the conditions under which G2 is precipitated, at pH 5·0 and low ionic strength, are similar to those used to isolate fibrinolytic enzymes (Milstone, 1941). The distinction between PF and fibrinolysins was made by comparing the two activities in several different preparations of G2 and other serum fractions, using human fibrinogen as the substrate for the fibrinogenolysins.

Comparative assays of the two activities in various fractions of guinea-pig serum establish quite clearly that fibrinogenolysin and permeability factors vary independently, according to the method of fractionation, and cannot be considered identical. This is evident in Table III*a*, where the results with three batches of G2 are set out. In each series ascending PF potency is associated with ascending fibrinogen life, *i.e.*, with descending fibrinogenolytic activity.

Similar results were obtained by Dr. Oscar D. Ratnoff with the fractions AP, G2α and G2β of Batch 16. Tested with bovine fibrin, G2β contained about 4 times as many Loomis fibrinolytic units as G2α; AP weakly inhibited bovine plasmin (Loomis) and human fibrinolysin activated by streptokinase. Contrary to our results with G2α from a batch fractionated in a different buffer, G2α was caseinolytic, but less so than G2β; AP was not.

All the G2 solutions tested were fibrinogenolytic, the fibrinogen life varying from 1–180 min. There was no activity in G3 or AP. We tried to separate the fibrinogenolytic and permeability activities, taking advantage of Kekwick and

Mackay's (1954) observation that most of the fibrinolysin in human plasma was precipitated at pH 5·3 and 10 per cent (v/v) ether. Serum batch 19 was accordingly divided into two portions. From one, G2 was prepared by the standard technique. From the other, a fraction P was precipitated at pH 5·3, I = 0·035 and the supernatant treated as for the isolation of G2, yielding a second fraction (G2–P). The fraction P, which represented 8 per cent of total serum protein, was actively fibrinogenolytic ; and in comparison with G2 (Table III*b*), the (G2–P) fraction had lost most of its fibrinogenolytic activity. It had also lost some PF, but to a much lesser extent. Clearly the separation was unsuccessful, but here again the absence of any direct association between the ratios of PF and fibrinogenolytic potency in the different fractions disproves the identity of PF and fibrinogenolysin.

This quantitative distinction was obtained with human fibrinogen. To eliminate the possibility of a species difference in susceptibility of human and guinea-pig fibrinogen to the guinea-pig enzymes, the test of Batch 22 was repeated with a specimen of guinea-pig fibrinogen (0·05 per cent) made by the method of Kekwick and Mackay (1954), with similar results (Table III*a*).

TABLE III.—*Comparison of Fibrinogen Life (Inversely Proportional to Fibrinogenolytic Activity) and Relative PF Potency of Various Serum Fractions.*

	Batch No.	Fraction.	Amount of fraction (mg.).	Fibrinogen life (min.). Human fibrinogen.	Fibrinogen life (min.). Guinea-pig fibrinogen.	Relative PF potency.
a	16*	G2β	0·1	15	nt	1
		G2	0·1	30	nt	9
		G2α	0·1	60	nt	21
	22*	G2β	1·0	40	<1/2	1
		G2	1·0	135	< 3	9
		G2α	1·0	625	> 60	24
	23*	G2β	1·0	24	nt	1
		G2	1·0	132	nt	8
		G2α	1·0	> 210	nt	21
b	18	G2-P	1·0	> 180	nt	1
		P	1·0	4	nt	4·8
	19	G2	1·0	1	nt	5·3
		P	1·0	1	nt	3·6
		G2-P	1·0	135	nt	1·0

* Electrophoretic separation of G2α and G2β in phosphate buffer, pH 8·0, for batch 16 ; and in glycine buffer, pH 9·0, for batches 22 and 23.

nt = not tested.

The distinction between PF and fibrinogenolysin was confirmed by tests of heat stability. The guinea-pig fibrinogenolysin, like the human (Macfarlane and Biggs, 1948) is inactivated by moderate heat. A fibrinogenolysin fraction was separated from a serum pool by precipitation at pH 5·2 (Milstone, 1941). In a saline solution containing 111 μg./ml., heating for 30 min. at 55° increased the fibrinogen life from 32 to 420 min. ; in contradistinction, as Fig. 9 shows, the PF potency of this fraction was unaffected.

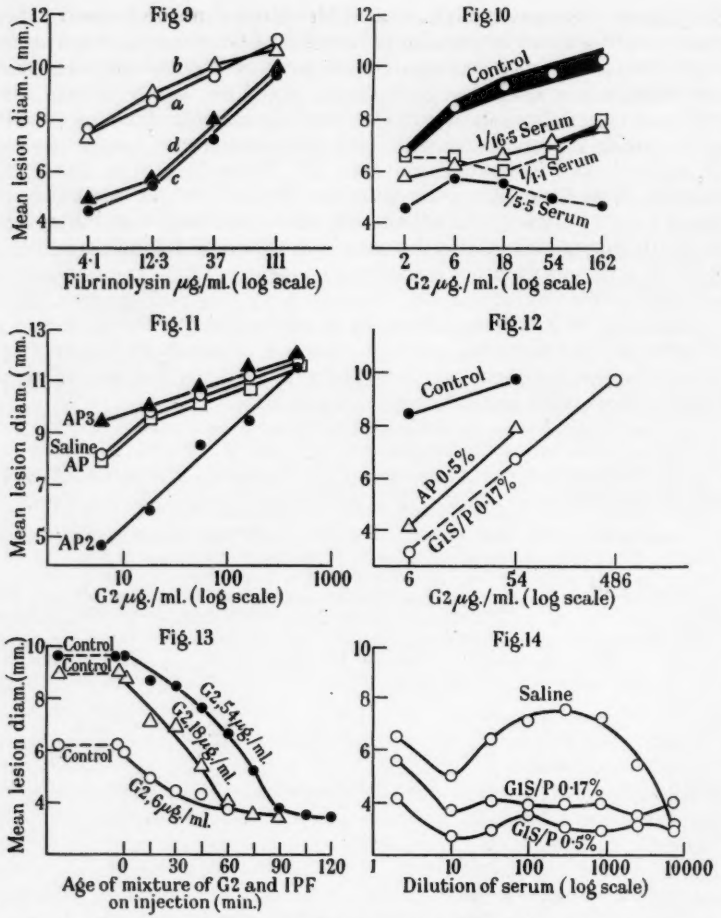


FIG. 9.—The distinction between PF and fibrinogenolytic activity of a fraction (see text) by susceptibility to heat and soya bean trypsin inhibitor.

a = unheated fibrinogenolysin.
b = heat-inactivated fibrinogenolysin, after 30 min at 55°.
c = unheated fibrinogenolysin + soya bean trypsin inhibitor.
d = heat-inactivated fibrinogenolysin + soya bean trypsin inhibitor.

FIG. 10.—The inhibition of PF/G2 by the inhibitor, IPF, in fresh serum. Ascending intensity of lesion blueing was graded trace, ±, +, +±, ++ up to ++++. The thickness of lines joining points is proportional to mean colour intensity of lesions; broken lines indicate trace of colour only.

FIG. 11.—Relative PF-inhibiting potency of the albumin fractions AP, AP2 and AP3.

FIG. 12.—Relative PF-inhibiting potency of AP and G1S/P.
(Broken line = plot from another assay.)

FIG. 13.—The inhibition of PF/G2 by the inhibitor in serum fraction AP.

FIG. 14.—The inhibition of PF/Dil., as activated in serial dilutions of fresh serum, by the inhibitor in serum fraction G1S/P. (Serum dilutions are reciprocals.)

In summary, our results indicate that PF is not a fibrinogenolysin or fibrinolysin ; they suggest that the hypotensive effect of G2 preparations in the guinea-pig is due to PF, and, though we have so far failed to discover any *in vitro* enzymic action, that PF is a protease.

The Isolation of IPF, the Serum Inhibitor of PF.

We have already postulated the presence in serum of a substance, IPF, which combines slowly with PF/Dil and inhibits it (Miles and Wilhelm, 1954). Preparations of PF/Dil activated by dilution of the serum to 1/135 are unsatisfactory as indicators of IPF activity. They probably contain some active IPF, and their potency slowly changes with ageing, even at 2°. For testing IPF potency we therefore used as our test permeability factor the more stable PF/G2, which keeps indefinitely at — 10°. In adopting this course we anticipate a conclusion reached later in this paper, namely that PF/Dil and PF/G2 are identical. For the present purpose the most important criterion of identity of the two is that both are inhibited by the IPF in guinea-pig serum.

The inhibition of PF/G2 by serum IPF.—Saline dilutions of a specimen of fresh serum which had stood for 90 min. on the bench exhibited the usual absence of blueing at 1/1, 1/5, and 1/15 when titrated in the blued animals. Nine volumes of these concentrations were added, each to 1 volume of a series of graded concentrations of G2, so as to give final G2 concentrations of 2 to 162 μg./ml. in serum dilutions of 1/1·1, 1/1·5 and 1/16·5; *i.e.*, dilutions still in the non-blueing range.

The mixtures were held for 30 min. at room temperature before testing. In the plotted results (Fig. 10) both intensity of blueing and lesion diameter are represented. The curves are difficult to interpret in detail but it is clear that, like PF/Dil, G2 is inhibited by the IPF in undiluted serum, and that, as in tests with PF/Dil, more IPF is activated with dilution of the serum to 1/5.

Fractionation of serum for IPF.—A single batch of G2 was used throughout for testing IPF activity. Unless otherwise stated, all inhibitor fractions were tested in a concentration of 0·5 per cent protein, and held with G2 for 45 min. at room temperature before injection. Their potency was estimated in terms of the amount of G2 inhibited.

Other serum inhibitors (Macfarlane and Biggs, 1948) are usually found among the more soluble proteins associated with the albumin fraction, and our first tests were accordingly made on the fraction AP, which is 88–96 per cent albumin and 4–12 per cent α-globulin. Some batches of AP contained an IPF, whereas others had only PF activity. AP was purified as follows. The fraction AP1, containing residual α_2-globulin, was removed and discarded. AP2, precipitated from the supernatant of AP1, was found to be an active inhibitor ; AP3, made from the AP2 supernatant, was uniformly inactive. AP2 was 94–96 per cent albumin, AP3 was electrophoretically homogeneous albumin (Fig. 1(*b*) and Table IV). The IPF activity of these fractions (Fig. 11) was therefore associated with the presence of α_1-globulin in the albumin fraction. Analysis of the electrophoretic patterns of a series of IPF fractions showed that when the approximate ratio of the α_1- and α_2-globulins dropped below 7:1, there was not sufficient inhibitor to overcome the α_2 PF which contaminates AP fractions (see Table IV), and the preparations induced blueing.

TABLE IV.—*Protein Composition of Fractions with IPF Activity.*

Batch of serum.	Fraction.	Percentage protein content.				Nitrogen (mg.).			
		Alb.	Globulin. α_1	Globulin. α_2	Globulin. β	Total.	α_1	α_2	$\alpha_1 : \alpha_2$
Pool of 12, 13, 15	AP	96	3	1	—	4,400	132	44	3 : 1
	AP2	92	8	—	—	1,010	81	—	—
	AP3	100	—	—	—	1,600	—	—	—
16	AP	96	4	—	—	nt	nt	nt	nt
17	„	89·8	9	1·2	—	640	57·5	7·7	7 : 1
24	„	92	6	2	—	1,285	77	25·6	3 : 1
17	G1S/P	2	91	7	—	91	83	6·4	13 : 1
22	„	4	87	9	—	577	505	52	10 : 1
23	„	—	90	6	4	1,400	1,260	84	15 : 1

nt = not tested.

Grob (1949–50) and Lyttleton (1954) also found anti-enzyme activity in fractions from human serum which were rich in α-globulin. Lyttleton prepared this fraction from the supernatant (G1S) left after precipitating the G1 fraction. We made a similar fraction, G1S/P, from guinea-pig serum. Both electrophoretic and ultracentrifugal analysis showed this to be 95–98 per cent α-globulin, of which 87–91 per cent was α_1. It represented 10·5 per cent of the G1S protein, and contained less than 5 mg./100 ml. cholesterol. In phosphate buffer pH 8·0 it formed a clear orange solution, the pigment travelling electrophoretically with the main component.

Fig. 12 shows a comparison of the IPF activity of the relatively PF-free specimen of AP, and of the G1S/P (for composition see Table IV), both of which were prepared from aliquots of G1S, Batch 17. Only the mean lesion diameters within the range 4–10 mm. are plotted, higher values in the flattening part of the dosage-response curve being omitted. Taking an 8 mm. lesion diameter as a convenient response for comparison, it can be estimated from the position of the two slopes that 0·17 per cent G1S/P has about 2·5 times the inhibiting potency of 0·5 per cent AP, indicating that G1S/P is about 7 times as potent as an equal concentration of AP. These estimates are approximate only, but were similar in repeated experiments, indicating that G1S/P fractions had more than 5 times the inhibitory potency of the best preparations of AP. We have not purified IPF beyond this stage. The properties of IPF described in the next section were examined in both G1S/P and AP fractions.

Properties of IPF.

The evidence from the fractionation strongly suggests that IPF is an α-globulin. Electrophoretically it differs from PF in moving more with the α_1 than the α_2 component; unlike PF, which is associated with lipoprotein globulins, IPF is associated with non-lipid globulins. This is evident from a comparison of the cholesterol content of potent preparations of PF and IPF (Table I).

Stability to cold, heat and pH.—AP fractions with high IPF activity, in 3–6 per cent solution in phosphate buffer at pH 8, maintain their original activity only for about 4 weeks at − 10°. For example, a fresh preparation, of which the standard dose inhibited 94 per cent of a fixed amount of G2, when frozen at − 10°, inhibited 91 per cent after 3 weeks, 77 per cent after 6 and < 5 per cent after 10 weeks. Loss of inhibitor activity is accompanied by rising PF activity

in the AP fractions, as though with destruction of IPF some PF had been released from an (IPF + PF) complex. Attempts to circumvent the instability in solution, by freeze-drying from pH 8 phosphate buffer, were unsuccessful; the freeze-dried preparations had lost all IPF activity. However, preparations of G1S/P were more stable; the IPF activity is preserved for at least 20 weeks at − 10°.

As regards heat stability, it was found that 0·5 per cent solutions in saline of both G1S/P and AP, when heated for 30 min. at 65°, lost all IPF activity.

The stability to pH was determined by bringing solutions of a G1S/P fraction to pH 4 and pH 5 with 0·5 M acetic acid and to pH 9 with 0·2 M-NaOH; after 3 days at 2° the solutions were dialysed for 3 days against repeated changes of phosphate buffer, pH 8, and 1 per cent concentrations then held for 45 min. at room temperature with equal volumes of G2. Three such experiments on the same batch of G1S/P indicated that IPF is to some extent labile at pH 4, and most stable at pH 5. The difference at all pH levels was not however very great.

Biological properties.—Preparations of IPF have a relatively low potency, and, in sharp contrast to SBTI, act slowly. These facts are evident in the experiment recorded in Fig. 13. Mixtures of equal volumes of 0·5 per cent AP and solutions of G2 were held at room temperature for periods of 1–120 min. before intracutaneous injection. With a constant amount of IPF, sufficient to inactivate the largest amount of PF used after 2 hr., the inactivation is progressive, and its rate is roughly proportional to the concentration of G2 in the mixture. The 5 mg./ml. solution of AP, which represents about 1 mg./ml. of G1S/P, inactivates as little as 6 μg./ml. G2 in 30 min., and only 54 μg./ml. in 90 min.

The HF in G2 is also inactivated. The action of G2, 100 μg./kg., on guinea-pig blood pressure, was completely abolished by prior incubation of G2 for 45 min. at room temperature with 20 times its weight of fraction G1S/P; similar incubation with a purified albumin (AP3) did not affect the HF.

That the IPF in the fraction G1S/P is the same as in low dilutions of serum is evident from its inhibitory action on PF/Dil. Dilutions of a fresh serum were activated by holding for 1½ hr. at room temperature, and divided into 3 sets. Equal volumes of saline, of 0·33 per cent and of 1·0 per cent G1S/P respectively, were added to each set of dilutions, and each of the individual mixtures injected after 45 min. at room temperature. The peak of PF/Dil activity is completely flattened by a final concentration of 0·17 per cent G1S/P (Fig. 14).

The IPF in the fraction G1S/P in concentrations up to 1 per cent does not alter the permeability effects of histamine, 5 and 50 μg./ml. or of the histamine-liberator, 48/80, 5 and 50 μg./ml.; but it slightly inhibits those of leukotaxine, 100 μg./ml.

The Relations of the Permeability Factors in Fresh Serum and in Serum Fractions.

The amount of PF/Dil activable by dilution of fresh serum can be measured in terms of the effective blueing dose (EBD), defined as the amount in 0·1 ml. producing a mean lesion diameter of 6 mm. On this basis, serum contains some 20,000 EBD/ml. of PF/Dil activated in dilutions 1 hr. old; peak activation occurs at a dilution of about 1/200, which therefore contains about 100 EBD/ml. (Miles and Wilhelm, 1954). The serum EBD content of PF/G2α is determined as follows. Guinea-pig serum contains 45 mg./ml. protein, of which 11 per cent is α_2-globulin. G2 contains 18 per cent of the serum α-globulins; all are α_2-

globulin and all are segregated in G2α (Table V). One ml. of serum therefore contains $18 \times 11 \times 45/10^4 = 891$ μg. G2α (*i.e.*, nearly 1 mg./ml.); and since the average EBD of good preparations of G2α is 0·04 μg. in 0·1 ml., 1 ml. serum contains 22,000 EBD of G2α. The slopes of the dosage-response of PF/G2α and PF/Dil have the same approximate magnitude (3·5–4·0), so that their EBD values are directly comparable. The value 22,000 obtained by fractionation is

TABLE V.—*Alpha-globulin Content of Fractions of Guinea-pig Serum* (*Batch* 23).

Fraction.	Nitrogen (mg.). Total.	a_1	a_2	$a_1 + a_2$	Percentage of total α-globulin.
Serum	17,100	1,592	1,880	3,472	100
G2	1,395	—	640	640	18
G2/2*	1,340	—	405	405	12
G1S/P	1,400	1,260	84	1,344	39
AP	4,240	360	—	360	10
G3	1,460	—	161	161	5

* G2/2 is a subfraction of G2 with negligible PF and IPF activity.

close to the value of 20,000 for PF activated by dilution in 1 hr., and is far in excess of the only other PF found in serum, namely PF/Age, of which a maximum of 80 EBD/ml. appear in old sera. These facts, the relative insusceptibility of PF/Age to IPF and SBTI, and the derivation of PF/G2α from *fresh* serum, indicate that PF/G2α is certainly not PF/Age, and is almost certainly PF/Dil.

The identity of PF/G2α and PF/Dil is also evident from their similar susceptibility to various inhibitors. Both are inhibited by serum IPF, the IPF in the fraction G1S/P, and by SBTI; both are moderately susceptible to PTI; and neither is susceptible to mepyramine maleate. Moreover, measured in EBD, both are inhibited to approximately the same degree. At peak activation, after 1 hr., in dilutions of 1/135 to 1/200, serum contains 150–100 EBD/ml. of PF/Dil; 150 EBD of PF/Dil is equivalent to 6 μg. of PF/G2α. Collecting the results of inhibition tests recorded in this and our first paper, we have as follows:

Inhibitor	Concentration inhibiting: PF/Dil (100–150 EBD/ml.)	PF/G2 (6 μg./ml.)
IPF (serum)	1/5 dilution	1/5 dilution
IPF (G1S/P)	1·7 mg./ml.	1·7 mg./ml.
SBTI	4–5 μg./ml.	6 μg./ml.
PTI*	200 μg./ml.	486 μg./ml.

* Partial inhibition only.

Furthermore, PF/Dil and PF/G2α are both large-molecular substances present in plasma and serum; both increase the permeability of the skin capillaries at the same speed; in both cases the capillaries recover their normal low permeability within the same short period of 10–15 min. The small amount of PF/Dil, equivalent to about 5μg. PF/G2α, present in 1 ml. of serum maximally activated by dilution to 1/200, precludes any measurement of its hypotensive activity or power to induce tissue leucocytosis in the skin, because the doses of G2 that consistently induce these effects are of the order, respectively, of 100 μg./kg. and 400 μg./ml.

The outstanding difference between PF/Dil and PF/G2α is the greater heat lability of PF/Dil. This, however, may be justifiably regarded as accidental,

because active preparations of PF/Dil of necessity contain all the other serum proteins, and it is highly probable that during heating PF/Dil combines with other serum proteins, and with the IPF, to form inactive complexes (Miles and Wilhelm, 1954).

DISCUSSION.

The properties we described for PF/G2α and of the IPF fraction G1S/P are precisely those deducible from our analysis of the phenomenon of activation of PF/Dil in dilutions of serum, but we have as yet no clue to the mechanism whereby the PF is so readily activated *in vitro*, either by dilution or during the process of serum fractionation.

Our investigations have been confined to the elucidation of a laboratory phenomenon—the artificial activation in serum of a protein permeability factor, and its inhibition by other serum proteins. At present we are clearly in no position to estimate the importance of these substances in the vascular phenomena of inflammation. Nevertheless the history of another biological system, the plasma fibrinolysins and their inhibitors, whose elucidation also began with the investigation of the properties of blood *in vitro*, justifies at this stage of our work some speculative comments on the substances we describe.

Some generalisation from the guinea-pig is permissible, because permeability factors are present in the serum of all the mammals, including man, which we have tested (to be published), though their properties and the conditions of their activation appear to differ with the species. Moreover, the permeability factors of one species are effective in the tissues of other species. The guinea-pig PF, for example, not only induces blueing in the skin of the rat and rabbit, but induces whealing in the dog skin, and the typical wheal and flare reaction in human skin—the last phenomenon being obviously relevant to the identification of Lewis's H-substance. None the less, although this interspecies reactivity suggests that permeability factors, like complement and the fibrinolysins, are general and partly interchangeable features of mammalian sera, interspecies generalisations must be made with caution. Our finding that the guinea-pig α_2-globulin PF was hypotensive for guinea-pig, dog and cat, but that the non-PF β-globulins of the guinea-pig were hypotensive in the rabbit is a case in point.

Our tentative identification of the permeability factor as a protease rests on the fact that activity is consistently associated with a protein; that it is very highly active measured on a molar basis; that PF occurs in fractions of serum—the α-globulins—known to contain other enzymes; and that it is strongly inhibited by soya bean trypsin inhibitor and moderately so by pancreatic trypsin inhibitor, substances of which we can find no record that they inhibit enzymes other than proteases. We know neither its site of action nor its substrate *in vivo*, and we have found no *in vitro* substrate.

Until a substrate is found, the enzymic nature of the factor remains unproven, and all assays of the permeability factor must be made *in vivo*. The skin of the guinea-pig with circulating dye has proved to be an admirable tissue for consistent bio-assay, and estimates of potency can be made with some accuracy. If positive results due to species incompatibility are to be avoided, sera must be investigated in the homologous species. The rat and the rabbit have proved to be less useful in this respect, and the assay of human permeability factors in man presents obvious difficulties. Nevertheless, a reliable measure of potency is required if we

are to determine the significance of these factors in physiological and pathological processes. The fact that we can with some confidence say that guinea-pig plasma contains about 1 mg./ml. of the precursor of a readily activable permeability factor which, weight for weight, is rather more active than histamine, gives that factor a better claim than histamine as a natural mediator of changes in permeability, at least on the grounds of amount and of availability in the capillaries. Histamine also takes second place in that so few of the pathological increases in permeability, and those mainly among the acute allergies, are susceptible to antihistaminic drugs—in this respect resembling the permeability induced by the serum permeability factor.

The claims of leukotaxine as a mediator of early inflammation are difficult to assess in the absence of any precise data on the rapidity and conditions of its formation in situations other than acute inflammatory exudates, and on the amounts formed. It may be argued that proteolysis to the small polypeptide stage, which is usually postulated as the origin of leukotaxine, is a slower and a more pathological process than activation of a permeability factor like the PF; but there is no reason why a rapid proteolysis should not occur in the early stages of inflammation. But, here again, the availability of PF in the tissues gives it a prior claim; and, weight for weight, the PF was more powerful than our preparation of leukotaxine in increasing capillary permeability, and as powerful in making capillary walls sticky and initiating a tissue leukocytosis. Menkin's (1951) "exudin" is insufficiently defined for identification with PF. It resembles the PF in being a large molecular substance, and it is precipitable from acid inflammatory exudates by concentrations of ammonium sulphate which would bring down euglobulins from serum. It differs in being heat labile; but the heat lability of active factors in mixtures with proteins and other large molecular substances cannot be regarded as definitive.

The participation of proteases, and especially of fibrinolysins, in various kinds of inflammation has been suggested by several investigators. In Ungar's (1952) view, they may be responsible for the liberation of both histamine and leukotaxine, which he regards as ultimate mediators of the vascular events of inflammation. The guinea-pig PF fails to fit this concept in two important aspects. It is certainly not a fibrinolysin (indeed, guinea-pig fibrinolysin does not increase capillary permeability in guinea-pigs), and almost certainly it does not liberate histamine. There is of course no necessity to postulate small molecular mediators between PF and its permeability effects; it may act directly on the endothelial cells of the blood capillaries, on the cement substance, or on the protein layer lining the inner surface of the endothelium postulated by Danielli (1940; see also Chambers and Zweifach, 1947).

It is possible that the effects ascribed to plasma fibrinolysins in inflammatory reactions (see reviews by Macfarlane and Biggs, 1948; and by Ungar, 1952, 1953) may sometimes have been due to associated proteins like PF. Both, for example, are activable by dilution, and in guinea-pig serum the two are separable only with great difficulty. Moreover, their respective serum inhibitors both occur in albumin fractions containing α_1-globulins.

The systemic reactions to PF, of which hypotension is the most striking, and their inhibition by IPF clearly demand investigation of the rôle of these two blood substances in the various forms of hypotensive shock, both in terms of the activation of PF, or of the destruction of the native inhibitor.

SUMMARY.

Guinea-pig serum contains the inactive precursor of an α_2-globulin, which on dilution of the serum in 0·85 per cent saline, or during its fractionation in aqueous ethyl-ether systems, is transformed into a powerful, relatively heat-stable permeability factor, PF. About 1 mg. of highly active preparation can be obtained from 1 ml. serum.

On intracutaneous injection in the guinea-pig, maximally active preparations of PF are vaso-dilator and rapidly increase capillary permeability, about as effectively as histamine on a weight basis, and $>$ 2000 times as effectively on a molar basis. PF also makes the inner wall of the capillaries sticky, and in larger doses promotes the diapedesis of blood leucocytes. There is no evidence that these effects are produced by the liberation of histamine.

Preparations of the PF, in intravenous doses of 5–10 mg./kg., cause in the guinea-pig a non-fatal shock, with a general increase in the permeability of the skin capillaries. In doses of 0·1–1 mg./kg., according to the animal, they induce a temporary hypotension in the guinea-pig, cat and dog. This effect is not due to histamine, acetylcholine or serotonin, nor is it the same as the Brodie phenomenon.

The high molar activity of PF suggests that it is an enzyme. It is distinct from the serum fibrinolysin of the guinea-pig. So far no *in vitro* substrate has been found, but its ready inhibition by soya bean trypsin inhibitor suggests that PF is a protease.

Guinea-pig serum also contains a slowly-acting heat labile substance IPF, which inhibits the permeability factor. IPF is an α_1-globulin, the best preparations of which inhibit about 1/800th their weight of PF α_2-globulin preparations after 30 min. at room temperature.

Neither soya bean trypsin inhibitor nor the serum inhibitor IPF antagonises the induction of increased capillary permeability by histamine or the synthetic histamine-liberator 48/80. Leucotaxine is unaffected by the trypsin inhibitor, but slightly antagonised by the IPF.

The amount of PF activable in plasma, the close association of its precursor with the blood-vessel walls, and the readiness with which it is activated, suggest that it may play a natural rôle in the local vascular phenomena of inflammation, and perhaps in acute pathological states associated with hypotension.

The investigations of the hypotensive and histamine-liberating effects of serum fractions were carried out by Dr. M. Schachter.

We are indebted to Drs. I. H. Lepow, L. Pillemer and O. D. Ratnoff, Western Reserve University, Cleveland, Ohio, for their examination of serum fractions; to Professor W. T. J. Morgan for specimens of blood group substances; to Mrs. I. Batty for azocoll; to Dr. R. A. Kekwick for help in the interpretation of electrophoretic and ultracentrifugal data; and to the Nuffield Trust for its financial help in the prosecution of this work.

REFERENCES.

Brodie, T. G.—(1900) *J. Physiol.*, **26**, 48.
Cappell, D. F.—(1929) *J. Path. Bact.*, **32**, 595.
Chambers, R. and Zweifach, B. W.—(1947) *Physiol. Rev.*, **27**, 436.

CLARK, W. M.—(1928) 'The determination of hydrogen ions.' Baltimore (Williams & Wilkins), p. 206.
DANIELLI, J. F.—(1940) *J. Physiol.*, **98**, 109.
FELDBERG, W. AND MILES, A. A.—(1953) *Ibid.*, **120**, 205.
Idem AND PATON, W. D. M.—(1951) *Ibid.*, **114**, 490.
Idem AND SCHACHTER, M.—(1952) *Ibid.*, **118**, 124.
GOMORI, G.—(1936) *Amer. J. Path.*, **12**, 655.
GROB, D.—(1949–50) *J. gen. Physiol.*, **33**, 103.
HARDY, W. B. AND GARDINER, S.—(1910) *J. Physiol.*, **40**, Proc. lxviii.
KEKWICK, R. A., LYTTLETON, J. W., BREWER, E. AND DREBLOW, E. S.—(1951) *Biochem. J.*, **49**, 253.
Idem AND MACKAY, MARGARET E.—(1954) *Spec. Rep. Ser. med. Res. Coun. Lond.*, No. 286 (H.M.S.O.).
LYTTLETON, J. W.—(1954) *Biochem. J.*, **58**, 8.
MCFARLANE, A. S.—(1942) *Ibid.*, **149**, 439.
MACFARLANE, R. G. AND BIGGS, ROSEMARY—(1948) *Blood*, **3**, 1167.
Idem AND PILLING, J.—(1946) *Lancet*, ii, 562.
MACKAY, MARGARET E., MILES, A. A., SCHACHTER, M. AND WILHELM, D. L.—(1953) *Nature, Lond.*, **172**, 714.
MENKIN, V.—(1946) *Arch. Path.*, **41**, 376.—(1951) *Amer. J. Physiol.*, **166**, 509.
MILES, A. A. AND MILES, E. M.—(1952) *J. Physiol.*, **118**, 228.
Idem AND WILHELM, D. L.—(1955) *Brit. J. exp. Path.*, **36**, 71.
MILSTONE, H.—(1941) *J. Immunol.*, **42**, 109.
OAKLEY, C. L., WARRACK, G. HARRIET AND VAN HEYNINGEN, W. E.—(1946) *J. Path. Bact.*, **58**, 229.
ONCLEY, J. L., SCATCHARD, G. AND BROWN, A.—(1947) *J. Phys. Chem.*, **51**, 184.
PATON, W. D. M.—(1951) *Brit. J. Pharmacol.*, **6**, 499.
RATNOFF, O. D.—(1953) *J. clin. Invest.*, **32**, 473.
SCHACHTER, M. AND TALESNIK, J.—(1952) *J. Physiol.*, **118**, 258.
SCHILD, H. O.—(1939) *Ibid.*, **95**, 393.
SCHNEIDER, J. A. AND YONKMAN, F. F.—(1953) *Amer. J. Physiol.*, **174**, 127.
UNGAR, G.—(1952) *Lancet*, ii, 742.—(1953) *Int. Arch. Allergy*, **4**, 258.

THE ANTIVIRAL AND SYNERGIC ACTIONS OF ISATIN THIOSEMICARBAZONE AND CERTAIN PHENOXYPYRIMIDINES IN VACCINIA INFECTION IN MICE.

D. J. BAUER.

From the Wellcome Laboratories of Tropical Medicine, Euston Road, London, N.W.1.

Received for publication November 19, 1954.

THE chemotherapeutic activity of thiosemicarbazones in vaccinia virus infections was discovered by Hamre, Bernstein and Donovick (1950), who found that *p*-aminobenzaldehyde-3-thiosemicarbazone inoculated into the yolk sac would prolong the survival time of chick embryos infected with vaccinia virus by the same route on the 6th day of incubation; a similar effect was noted in mice infected with vaccinia virus intranasally and given the compound subcutaneously and by mouth. Thompson, Price and Minton (1951) found that benzaldehyde thiosemicarbazone had a much greater activity, since mice inoculated with up to 1000 LD_{50} of vaccinia virus intracerebrally and given a diet containing up to 0·08 per cent of the compound showed a significant degree of survival, amounting in some experiments to 12 out of 18 mice inoculated compared with 3 of 18 mice in a control group. In further work (Thompson Minton, Officer and Hitchings, 1953) numerous thiosemicarbazones were examined for activity against vaccinia infection in mice; the greatest effect was seen with the thiosemicarbazones of isatin, cinchoninaldehyde and 2-thenaldehyde. A number of 5-phenoxythiouracils were also similarly examined (Thompson, Price, Minton, Falco and Hitchings, 1951); 5-(2′ : 4′-dichlorophenoxy)thiouracil was the most active, but was not so active as the more active thiosemicarbazones.

In the present work an account is given of an independent investigation of the anti-vaccinial effect of many of the thiosemicarbazones and phenoxypyrimidines examined by Thompson and his collaborators; the results are in general agreement, but show in addition that the antiviral activity of isatin thiosemicarbazone is very much greater than that found in Thompson's experiments, and that the antiviral effects of isatin thiosemicarbazone and of certain phenoxypyrimidines are synergic, so that the activity of an appropriate mixture of the two compounds is much greater than the added effects which would be produced by the same doses of the two compounds given alone.

METHODS.

All experiments were carried out with the IHD strain of mouse-adapted vaccinia virus, which was kindly supplied by Dr. Randall Thompson of the University of Indiana. A 1 per cent extract of infected mouse brain in 70 per cent glycerol buffered at pH 7 was stored at −20°; Swiss white mice were inoculated with a suitable dilution of this material, and the brains were harvested after the appearance of symptoms and used as the source of virus for therapeutic tests. A 10 per cent suspension of infected brain was made in 10 per cent horse

serum containing 0·0066 M phosphate buffer of pH 7, and decimal dilutions up to 10^{-5} were made in the same diluent; 2 groups of 6 mice were inoculated intracerebrally with each dilution and assigned to test and control groups respectively. The compounds examined were all insoluble in water, and were suspended in saline with the aid of gum arabic; the concentrations were adjusted so that the required dose was contained in 0·1 ml., and mice in the test group were given 2 subcutaneous injections daily, beginning a few hours after inoculation with virus, and continuing to a total of 9 doses. Mice in the test and control groups were observed for a total period of 14 days and deaths were recorded. The LD_{50} was calculated for both groups; in the control group this figure represented the titre of virus present in the original preparation, and in the test group it gave a numerical measure of the effect produced by the treatment. The difference between the true titre in log units in the control group and the observed titre in the treated group gave the protection index, and was used as the final figure for the assessment of antiviral effect.

RESULTS.

Dose-response Curve of Isatin Thiosemicarbazone.

Several thiosemicarbazones were found to have antiviral activity in preliminary tests; the results were in general agreement with those of Thompson and co-workers, and will therefore not be reported here. An unexpectedly high degree of activity was found with isatin thiosemicarbazone, however, and a detailed investigation was therefore made of the effect of this compound. The results of two typical experiments are shown in Table I, in which the results

TABLE I.—*Effect of Isatin Thiosemicarbazone on Vaccinia Infection in Mice.*

Dose.	Group.	Virus dilution. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	Titre (log units).	Protection index (log units).
2·5 mg.	Test	2/6	1/6	0/6	0/6	0/6	1·0	4·0
	Control	6/6	5/6	5/6	4/6	4/6	5·0	
1·25 mg.	Test	2/6	1/6	0/6	0/6	0/6	1·0	4·3
	Control	6/6	6/6	6/6	6/6	5/6	5·3	
0·16 per cent in diet*	Test	—	0/6	2/6	0/6	—	<2	>1·9
	Control	—	6/6	5/6	2/6	—	3·9	

* Results of Thompson, Minton, Officer and Hitchings (1953); titres and protection index calculated from the published data.

Numerator: Number of mice dying of vaccinia encephalitis.

Denominator: Number of mice in group.

of the most favourable experiment of Thompson, Minton *et al.* (1953) rearranged in a similar manner are shown below for comparison. It can be seen that when the test is extended to include doses of virus up to 100,000 LD_{50} a protection index of 4 log units can be observed. This amounts to a 99·99 per cent degree of protection; an antiviral effect of this magnitude has hitherto only been observed with penicillin and sulphonamides in the lymphogranuloma-psittacosis group of viruses. It therefore represents a major advance in the chemotherapy of virus infections, although it is unfortunately far from certain, for reasons to be discussed later, that isatin thiosemicarbazone will be of any value in the treatment of vaccinia and variola infections in man.

The effect of varying the dose of isatin thiosemicarbazone was investigated in similar experiments; the same schedule of dosing was employed and the

individual dose given ranged from 0·05 to 2·5 mg. The results are shown in Fig. 1. The protection index rises gradually from 1 to 4 log units as the dose is increased from 0·05 to 0·5 mg., and is not significantly increased by increasing the dose up to 2·5 mg., although the theoretical maximum protection index attainable is 5 log units.

The Effect of a Limited Number of Doses of Isatin Thiosemicarbazone.

Isatin thiosemicarbazone is insoluble in water, and most of the amount given remains at the site of inoculation, although the presence of a yellow colour in the urine and faeces shows that the compound is readily absorbed; it was therefore considered likely that an adequate blood concentration could be obtained by giving 1 or 2 doses only. This possibility was investigated by inoculating mice with a dose of virus lying between 100 and 1000 LD_{50}, and treating them in groups of 6 with 1 or 2 doses of isatin thiosemicarbazone. The individual doses ranged from 0·25 to 2·5 mg., and were given up to 24 hr. after infection; 4 mice were left untreated as a control group. The results of several such experiments are shown in Table II. From Exp. 1 and 2 it is seen that complete

TABLE II.—*The Effect of Limiting the Period of Treatment with Isatin Thiosemicarbazone.*

Exp. No.	Number of doses.	Time after infection in hours.	Dose in mg. 0.	0·25	0·5	1·0	1·5	2·0	2·5
			Mortality.						
1	1	3	4/4	6/6	2/6	2/6	3/6	5/6	2/6
2	1	3	4/4	5/6	3/6	3/6	3/6	4/6	2/6
3	2	1½, 18	4/4	2/6	0/6	1/6	0/6	0/6	0/6
4	2	18, 24	4/4	1/6	0/6	0/6	0/6	0/6	0/6
5	1	18	2/4*	2/6	0/6	0/6	0/5	0/6	0/6

* Morbidity 4/4.
Numerator: Number of mice dying of vaccinia encephalitis.
Denominator: Number of mice in group.

protection cannot be obtained with a single dose of as much as 2·5 mg. when given 3 hr. after infection; in Exp. 3 and 4 two doses of as little as 0·25 mg. gave better than 50 per cent protection when given up to 24 hr. after infection, and in Exp. 5 the same effect was obtained with a single dose of 0·25 mg. given after 18 hr. It is thus evident that a single dose of the compound exerts a depôt effect; this however extends for a limited period of time, since the degree of protection is greatly reduced if the dose is given too soon after infection.

Dose-response Curve of 5-(2′ : 4′-Dichlorophenoxy)thiouracil.

In a preliminary investigation of the antivaccinial action of a number of phenoxypyrimidines the compound 5-(2′ : 4′-dichlorophenoxy)thiouracil was selected for further study. The dose-response curve was determined and is shown in Fig. 1. No effect was produced when doses of 0·5 mg. were given according to the usual schedule, but a protection index of nearly 2 log units was obtained when the dose was increased to 2 mg. The activity of the compound

is thus lower than that of isatin thiosemicarbazone by about 100-fold. It is nevertheless of interest that antiviral activity should be possessed by two compounds of such different chemical constitution, and the possibility arises that the two compounds act in different ways and might therefore show a synergic in place of a merely additive effect if given together.

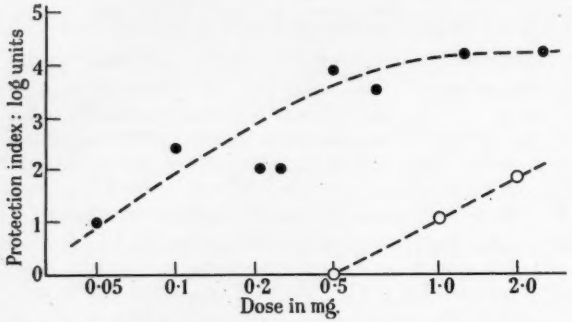


FIG. 1.—Dose-response curves of isatin thiosemicarbazone and 5-(2′ : 4′-dichlorophenoxy)thiouracil.
● isatin thiosemicarbazone
○ 5-(2′ : 4′-dichlorophenoxy)thiouracil.

Dose-response Curves of Various Combinations of Isatin Thiosemicarbazone and 5-(2′ : 4′: Dichlorophenoxy)thiouracil.

In order to detect a possible synergic effect between these two compounds, tests were carried out in which mice were given mixtures of various doses of isatin thiosemicarbazone combined with the phenoxythiouracil in increasing molecular proportions. The protection index was observed in the usual way and gave a family of dose-response curves when plotted against the molecular ratio of the two substances. Three such curves are shown in Fig. 2. Curve A was obtained in experiments with a 1·25 mg. dose of the thiosemicarbazone. The protection index rises to a peak of 5 log units when the two compounds are given in equimolecular proportions. This represents the full extent of the titre of the virus preparation used and is therefore the maximum degree of protection obtainable, corresponding to a reduction in mortality of $> 99{\cdot}999$ per cent. The difference between the protection index of 4 log units given by 1·25 mg. of isatin thiosemicarbazone alone and the theoretical maximum of 5 log units is not great enough to allow sufficient scope for the detection of synergism, and the experiments were therefore repeated with smaller doses. In the experiments of curve B the effect of a dose of 0·1 mg., which when given alone gives a protection index of 1·5 log units, was greatly increased by the simultaneous administration of the second compound, and attained a maximum of 4·5 log units with an equimolecular ratio. The effect of the dose of the thiosemicarbazone was thus increased 1000-fold by the simultaneous administration of a dose of 0·115 mg. of 5-(2′ : 4′-dichlorophenoxy)thiouracil, a dose which by itself is quite devoid of detectable activity, as can be seen from Fig. 1. The synergic action of the two compounds is thus clearly demonstrated. The same effect is seen in a more

marked degree in the experiment of curve C, where a dose of isatin thiosemicarbazone was used which when given alone produced a barely significant degree of protection; by the simultaneous administration of amounts of the phenoxythiouracil ranging up to 0·115 mg., a dose which is ineffective when given alone, the protection index could be increased over a 1000-fold range from 0·5 to 3·5 log units. The three curves all show a maximum near a molecular ratio of

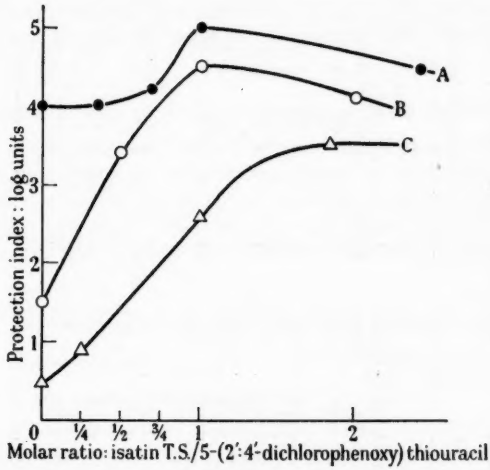


FIG. 2.—Dose-response curves of varying doses and molecular ratios of isatin thiosemicarbazone and 5-(2′ : 4′-dichlorophenoxy)thiouracil.

● 1·25 mg. isatin thiosemicarbazone.
○ 0·1 mg. „ „
△ 0·05 mg. „ „

unity. This might suggest that the two compounds act together in equimolecular proportions, but it should be borne in mind that they may be absorbed at different rates from the site of inoculation and may be present in the brain in entirely different proportions. The presence of a maximum does however imply that there is an optimum ratio of the two compounds at the site of action.

Synergic Effect of Analogues of 5-(2′ : 4′-*Dichlorophenoxy)thiouracil.*

A number of analogues of 5-(2′ : 4′-dichlorophenoxy)thiouracil were available for trial, and it was of interest to determine whether these also acted synergically with isatin thiosemicarbazone, and whether the possession of synergic activity was correlated in any way with the possession of antiviral activity when given alone. From a consideration of the results of Fig. 1 and 2 it was considered that synergism could be detected in a single experiment by combining a dose of 0·05 mg. of isatin thiosemicarbazone with an equimolecular proportion of the compound under examination; a protection index substantially in excess of the 0·5 to 1·0 log unit due to the thiosemicarbazone alone would then be taken as evidence of synergism. The results are shown in Table III. Synergic activity

TABLE III.—*Synergic and Anti-vaccinial Action of Certain Phenoxypyrimidines.*

Test of synergism.								Test of antivaccinial activity.				
Position of substituents in pyrimidine nucleus.			Titre.		Protection index.	Synergism.	Dose (mg.)	Titre.		Protection index.	Activity.	
2	4	5	Test.	Control				Test.	Control.			
SH	OH	2′ : 4′-dichlorophenoxy	2·4*	5·0*	2·6*	+	1	3·0*	4·0*	1·0*	+	
NH_2	SH	4′-chlorophenoxy	1·9*	5·1	3·2*	+	1	4·8*	5·0	0·2	−	
NH_2	NH_2	2′ : 4′-dichlorophenoxy	2·0	5·1	3·1	+	0·5	3·9*	5·0*	1·1*	+	
SH	OH	4′-methylphenoxy	1·1	3·9	2·8	+	2	4·5*	5·2	0·7*	+	
			2·1	5·3	3·2	+	—	—	—	—	—	
SH	OH	3′ : 4′-dichlorophenoxy	2·6*	5·4	2·8*	+	2·5	4·5*	⩾5·5	1·0*	+	
SH	OH	3′-methyl-4′-chlorophenoxy	⩾1·5	4·1	⩽2·6	+	2	4·5*	4·5*	0	−	
SH	OH	2′ : 4′-dibromophenoxy	2·9	4·8	1·9	±	2	4·0*	4·0*	0	−	
NH_2	SH	2′ : 4′-dichlorophenoxy	3·0*	5·0*	2·0*	±	1	5·0*	5·0*	0	−	
NH_2	NH_2	4′-chlorophenoxy	4·0*	4·5*	0·5*	−	1	4·5	5·1*	0·6*	+	
SH	OH	4′-chlorophenoxy	4·0*	5·0*	1·0*	−	2	5·1	⩾5·5	⩾0·4	+	
SH	OH	4′-bromophenoxy	4·0*	4·5*	0·5*	−	2	4·8*	5·0*	0·2	−	

* The titre could not be calculated exactly on account of survivals in the lower dilutions.

was found in 5 compounds; 2 compounds showed probable activity and 3 were negative. The series examined is too small to permit of valid conclusions concerning the structure necessary for synergism, but it appears that considerable variation in the substituents may occur without loss of synergism, and in the 2 : 4-diamino compound activity is retained in the absence of the SH group; in the thiosemicarbazones, on the other hand, the presence of sulphur is necessary for activity, since it has been shown by Thompson, Minton *et al.* (1953) that the corresponding semicarbazones are inactive. It also seems that synergism in the phenoxypyrimidines is not necessarily correlated with the possession of anti-vaccinial activity.

Immune State of Animals Surviving After Treatment.

The immune state of mice which had survived infection after being treated with isatin thiosemicarbazone was investigated by noting the response to an intracerebral inoculation of 100 LD_{50} of vaccinia virus given 18 days after the original inoculation of virus. The results of typical experiments are shown in Table IV, in which the numerators now indicate the number of mice which

TABLE IV.—*Immune State of Mice Surviving Infection after Treatment.*

	Initial infection.		Challenge infection of survivors from treated groups.	
Treatment: 2·5 mg. isatin thiosemicarbazone twice daily.				
Virus dilution.	Treated.	Controls.	Treated.	Challenge control.
10^{-4}	6/6	2/6	6/6	0/4
10^{-5}	6/6	2/6	6/6	
Treatment: 2·5 mg. isatin thiosemicarbazone + 1·25 mg. 5-(2′ : 4′ dichlorophenoxy)thiouracil twice daily				
Virus dilution.	Treated.	Controls.	Treated.	Challenge control.
10^{-2}	4/5	0/5	4/4	0/4
10^{-3}	6/6	2/6	6/6	
10^{-4}	5/5	2/6	5/5	
10^{-5}	6/6	2/6	6/6	

Numerator: Number of mice surviving infection.
Denominator: Number of mice in group.

survived. It will be seen that mice which survived infection with vaccinia virus as a result of treatment with isatin thiosemicarbazone alone or combined with 5-(2′ : 4′-dichlorophenoxy)thiouracil were in all cases solidly immune to reinfection. This finding was entirely unexpected; after the inoculation of a large dose of virus (10^{-2} dilution) some degree of multiplication might well occur even in the presence of adequate treatment, and might lead to immunity, but if so, the same treatment should entirely suppress the multiplication of a much smaller amount of virus (10^{-5} dilution) and no immunity should develop. The findings are entirely at variance with such predictions, and it must be concluded that in spite of the apparent success of the treatment the virus multiplies in all cases to an extent sufficient to produce immunity.

Effect of Treatment on Multiplication of the Virus.

Mice were inoculated intracerebrally with a dose of vaccinia virus ranging from 10 to 1000 LD_{50} and were then assigned to two groups, one of which was treated in the usual manner. At daily intervals after infection brains were removed from two mice from both treated and control groups and the content of virus was titrated by the subinoculation of mice with serial dilutions of the pooled extracts. From the results of the titrations the rate of development of virus could be determined. Representative experiments are shown in Table V.

TABLE V.—*Effect of Chemotherapy on Multiplication of Vaccinia Virus in Mouse Brain.*

Treatment.	Virus dose LD_{50}	Day.	Titre. Treated.	Titre. Control.
Isatin thiosemicarbazone 2 mg. twice daily	10^3	1	0·75	2·5
		2	3·75	3·75
		3	4·25	⩾5·5
		4	⩾5·5	6·25
	10^2	1	? 3·5	3·25
		2	⩾4·5	⩾4·5
		3	5·5	⩾6·5
		4	5·25	⩾6·5
	10^4	1	1·5	3·25
		2	3·25	3·5
		3	4·75	6·25
Isatin T.S. 1 mg. and 5-(2′ : 4′-dichlorophenoxy)thiouracil 1·15 mg. twice daily	10^3	1	1·25	1·5
		2	*ca.* 4·0	2·75
		3	3·25	4·0
		4	3·5	5·0
Above compounds in doses of 1·25 and 1·44 mg. respectively	10^4	1	? 1	1·5
		2	? 1	3–4
		3	3	5·25
		4	⩾5·5	5

It will be seen that treatment with the thiosemicarbazone alone or combined with the phenoxythiouracil does not prevent the multiplication of the virus to a titre which may exceed 5·5 log units ; the titres in the treated animals, however, are usually 1 log unit lower than in the controls, and somewhat greater differences could sometimes be observed when the two compounds were given together. From a considerable amount of experimental work carried out with the mouse-adapted strain of vaccinia virus it has been found that symptoms of encephalitis will not appear until the virus titre exceeds a critical level which is somewhere around 6·5 log units. The protection conferred by treatment, although amounting only to about 1 log unit, evidently serves to keep the virus titre below the critical level for the appearance of symptoms, while at the same time sufficient growth of virus occurs to stimulate the production of antibody.

The Effect of Isatin Thiosemicarbazone on Other Viruses.

In view of the profound effect exerted by isatin thiosemicarbazone on the course of infection with vaccinia virus in mice it was of interest to determine

TABLE VI.—*Effect of Anti-vaccinial Compounds on Other Viruses.*

Virus.	Dose. Isatin T.S. (mg.).	Dose. 5-dichlorophenoxythiouracil. (mg.).	Titre. Test.	Titre. Control.	Protection index.	Activity.
NYF	1·25	2·5	⩾6·5	⩾6·5	0	None
NYF	2·5	—	⩾6·5	⩾6·5	0	,,
LCM	1·25	5·0	⩾6·5	⩾6·5	0	,,
RVF	2·5	—	5·1	⩾5·5	⩾0·4	Slight
RVF	2·0	—	4·0	4·5	0·5	,,
NWS	2·5	—	⩾6·5	6·1	0	None
Dengue	2·0	—	1·9	1·9	0	,,
MM	2·0	—	⩾6·5	⩾6·5	0	,,

Abbreviations: NYF, neurotropic yellow fever; LCM, lymphocytic choriomeningitis; RVF, Rift Valley fever.

whether it was effective against any other viruses. The results of such experiments are shown in Table VI. There was no evidence of any activity against neurotropic yellow fever, lymphocytic choriomeningitis, dengue, MM or the NWS strain of influenza virus, but there was some activity against Rift Valley fever, although not of the same order as that against vaccinia.

DISCUSSION.

It is evident that mortality due to vaccinia infection in mice can be almost entirely suppressed by isatin thiosemicarbazone. Treatment with 5-(2′ : 4′-dichlorophenoxy)thiouracil also confers a considerable degree of protection, and the two compounds given together act in synergism. This indicates that the mode of action of the two compounds is not the same. There is some evidence that a factor contributed by the host may enter into the active complex. For instance, the maximum degree of protection obtainable with isatin thiosemicarbazone is given by quite a small dose, and the effect cannot be increased by greatly increasing the dose; the compound, although insoluble, is adequately absorbed, and it is possible that the limitation of therapeutic effect is brought about by presence in the brain in fixed concentration of some substance which is essential to the activity of the thiosemicarbazone. It has been found by Thompson, Davis, Russell and Hitchings (1953) that isatin thiosemicarbazone has no activity against vaccinia virus in rabbits. The dose given was high enough to ensure adequate absorption, and the absence of effect could be explained by postulating that a host factor present in mice was lacking in rabbits or present in insufficient concentration. The host factor, if it exists, might be copper or some other metal which would form a chelate complex with thiosemicarbazones which might be the active compound. The same workers investigated the effect of giving salts of various metals in combination with isatin thiosemicarbazone, but they were trying to detect an inhibitory effect only, and their experiments were presumably not designed to detect the occurrence of synergism.

Have isatin thiosemicarbazone and 5-(2′ : 4′-dichlorophenoxy)thiouracil any clinical application? The lack of activity in rabbits does not preclude this, but the fact that the growth of virus in mice is not suppressed, but only retarded,

suggests that the effect would be less marked in a disease, such as smallpox, which is characterised by an exanthem, and that complete success in the chemotherapy of smallpox is not likely to be achieved until analogues or synergic combinations which actually suppress virus growth have been discovered by further work. However, one possible application has already been pointed out in the experimental section; the results of giving a limited number of doses of isatin thiosemicarbazone during the incubation period suggest that a single dose of the compound, which Thompson and his collaborators have shown to be active by mouth, might be expected to confer some degree of immediate protection to susceptible persons who have been exposed to smallpox, and the compound might also have some application as a prophylactic in epidemics. The toxicity of isatin thiosemicarbazone is low, and mice dosed for five days in succession show no ill effects, but it should be stressed that there would be a risk in introducing the compound into clinical use until much more extensive investigations of its toxicity have been carried out.

SUMMARY.

Treatment with isatin thiosemicarbazone in suitable doses will confer 99·99 per cent protection against mortality in mice infected intracerebrally with a neurotropic strain of vaccinia virus. The compound has a depôt effect and a high degree of protection can be obtained with a single dose of 0·25 mg. given 18 hours after infection.

Treatment with 5-(2′ : 4′-dichlorophenoxy)thiouracil confers protection, but to a much lesser extent.

Isatin thiosemicarbazone and 5-(2′ : 4′-dichlorophenoxy)thiouracil act synergically, since the combined effect of small doses of each given simultaneously is very much greater than the individual effects of the same doses given separately. Some other phenoxypyrimidines also act synergically with isatin thiosemicarbazone.

Mice surviving vaccinia infection as a result of treatment with isatin thiosemicarbazone are immune to reinfection.

Considerable multiplication of virus occurs in the brains of treated mice, but the titres attained are usually 1 log unit lower than in untreated mice, and do not reach the level at which symptoms usually appear.

Isatin thiosemicarbazone has a slight activity in infection with Rift Valley fever virus.

REFERENCES.

Hamre, D., Bernstein, J. and Donovick, R.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **73**, 275.

Thompson, R. L., Davis, J., Russell, P. B. and Hitchings, G. H.—(1953) *Ibid.*, **84**, 496.

Idem, Minton, S. A., Officer, J. E. and Hitchings, G. H.—(1953) *J. Immunol.*, **70**, 229.

Idem, Price, M. L. and Minton, S. A.—(1951) *Proc. Soc. exp. Biol., N.Y.*, **78**, 11.

Iidem, Falco, E. A. and Hitchings, G. H.—(1951) *J. Immunol.*, **67**, 483.

SURVEY OF PAPERS.

JENNINGS and FRANCES MALONE report further studies on zonal precipitin reactions using triangular agar plates, two corners of which are occupied by known antigen and antiserum, so that the presence of either in the blood of animals being immunised can be detected and roughly estimated (p. 1).

BLOCH and NOLL have studied the effect of cord factor derived from human tubercle bacilli on the course of tuberculosis in mice. They claim that a single injection causes both acute and chronic infections to progress more rapidly. Infections enhanced in this way respond less readily to the therapeutic action of isoniazid (p. 8).

ANDREWES and HARISIJADES have succeeded in passing myxoma virus serially through 30 transfers in the brains of day-old mice. No ill effects were produced and multiplication was shown by titrations in rabbit skin and on the chorio-allantoic membrane (p. 18).

JENIFER JOWSEY, MAUREEN OWEN, MARGARET TUTT and JANET VAUGHAN describe a continuation of their studies of the distribution of radio-active strontium in bone. Adult rabbits were used, and the total amount of strontium retained was found to be less than in young animals ; on the other hand, since it is deposited in sites of active growth, and these are much fewer in the adult, it is more highly concentrated in these sites. The rate of urinary excretion was studied and found to vary from day to day (p. 22).

LISCO and MARCIA R. WHITE have studied the protection afforded to mice given an intravenous injection of beryllium sulphate by administering aurintricarboxylic acid by the same route one hour later ; this substance does not, like BAL, hasten elimination, but activates beryllium *in situ* by forming an insoluble lake with it. The damage caused in the liver, spleen and bone marrow was shown histologically to be much reduced and transient : that in the renal tubules, although also transient, was somewhat enhanced when the larger of two doses of beryllium was given, one explanation for which may be the effect of a lower pH on the stability of the lake formed and on the solubility of beryllium (p. 27).

FONNESU and CLARA SEVERI have studied the accumulation of glycogen in the liver which occurs in poisoning with 2 : 4-dinitrophenol, and sought an explanation for it. The properties of this glycogen were indistinguishable from that in normal liver. Assay of various enzymes in such livers revealed no deficiency of those responsible for the conversion of glycogen to glucose. The effects of administering anterior pituitary somatotropic hormone showed that a deficiency of hyperglycaemic-glycogenolytic factor was not responsible. On the other hand, a much reduced glucose tolerance could be demonstrated, and it is suggested that decreased glucose utilisation in the tissues is responsible for the accumulation of glycogen in the liver (p. 35).

WINDRUM, KENT and EASTOE obtained reticulin by digesting the renal cortex of young subjects with weak alkali, and submitted the purified material to exhaustive chemical analysis, one object being to decide how reticulin differs from collagen. The protein component was shown to be composed of 19 amino acids, some of which were present in amounts greater or less than those stated to exist in collagen. Differences were greater in the carbohydrate and lipid content, the latter being unexpectedly high (10.9 per cent). The staining properties of reticulin are discussed in relation to these two components (p. 49).

HARPER has shown that 48 hours after exposure of guinea-pigs to infection with *Br. suis* by inhalation the organisms have begun to multiply. In the absence of evidence of invasion of lung tissue he suggests that multiplication takes place on the surface of lung epithelium,

that the bacteria are carried by phagocytes to lymph nodes, and that from these nodes infection reaches the general circulation. The further progress of the infection has been followed by quantitative cultural and other methods (p. 60).

MILES and WILHELM describe a permeability factor produced from diluted fresh guinea-pig serum which on intracutaneous injection into guinea-pigs rapidly induces an increased permeability of blood capillaries to circulating Pontamine Sky Blue. The factor is susceptible to soya bean trypsin inhibitor. A low potency inhibitor of this factor present in undiluted serum is also described (p. 71).

WILHELM, MILES and MARGARET MACKAY have isolated and examined the permeability factor described in the preceding paper. They find it to be an α_2-globulin normally occurring as an inactive precursor in guinea-pig serum. Dilution or fractionation in an ethyl-ether system transforms it into a relatively heat-stable permeability factor. About 1 mg. of highly active material can be obtained from 1 ml. of serum. This material is approximately equivalent in activity to histamine on a weight basis and $>$ 2000 times more active on a molar basis. The authors do not consider it to be related to histamine and suggest that because of its high molar activity it is an enzyme, possibly a protease. The substance present in guinea-pig serum inhibiting the permeability factor has been found to be an α_1-globulin (p. 82).

BAUER has found the capacity of isatin thiosemicarbazone to protect mice inoculated intracerebrally with a neurotropic strain of vaccinia virus much greater than has previously been reported. This drug does not prevent multiplication of the virus in the brain, but only reduces its degree just enough to prevent manifestations of the infection : treated mice are immune to re-infection. Isatin thiosemicarbazone acts synergically in this infection with a 5-(2′ : 4′-dichlorophenoxy)thiouracil, which alone has much less protective effect (p. 105).

Watson Stereoscopic Microscopes will satisfy the most fastidious user.

They are available in a wide range of stands and can be fitted with a variety of optics. Offering, as they do, a true stereoscopic image, they can be applied to any problem where the three dimensions, length, breadth and depth are to be investigated.

Write for List 6B.

The illustration is of the "Research" Model.

WATSON'S

W. WATSON & SONS LTD. 313 HIGH HOLBORN, LONDON, W.C.1.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXVI, NO. 1. FEBRUARY, 1955.

CONTENTS.

PAGE

JENNINGS, R. K. AND MALONE, FRANCES—THE DOUBLE DIFFUSION PRECIPITIN TECHNIQUE AS A TOOL FOR THE STUDY OF THE INDUCTION PERIOD OF ANTIBODY FORMATION 1

BLOCH, H. AND NÖLL, H.—STUDIES ON THE VIRULENCE OF TUBERCLE BACILLI. THE EFFECT OF CORD FACTOR ON MURINE TUBERCULOSIS 8

ANDREWES, C. H. AND HARISIJADES, S.—PROPAGATION OF MYXOMA VIRUS IN ONE-DAY-OLD MICE 18

JOWSEY, JENIFER, OWEN, MAUREEN, TUTT, MARGARET AND VAUGHAN, JANET—RETENTION AND EXCRETION OF ^{90}Sr BY ADULT RABBITS 22

LISCO, H. AND WHITE, MARCIA R.—THE MODIFICATION OF BERYLLIUM-INDUCED TISSUE DAMAGE IN MICE BY THERAPY WITH AURINTRICARBOXYLIC ACID 27

FONNESU, A. AND SEVERI, CLARA—GLYCOGEN ACCUMULATION IN THE LIVER DUE TO 2 : 4-DINITROPHENOL 35

WINDRUM, G. M., KENT, P. W. AND EASTOE, J. E.—THE CONSTITUTION OF HUMAN RENAL RETICULIN 49

HARPER, G. J.—*Brucella suis* INFECTION OF GUINEA-PIGS BY THE RESPIRATORY ROUTE 60

MILES, A. A. AND WILHELM, D. L.—ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION. I. AN ACTIVABLE PERMEABILITY FACTOR AND ITS INHIBITOR IN GUINEA-PIG SERUM 71

WILHELM, D. L., MILES, A. A. AND MACKAY, MARGARET E.—ENZYME-LIKE GLOBULINS FROM SERUM REPRODUCING THE VASCULAR PHENOMENA OF INFLAMMATION. II. ISOLATION AND PROPERTIES OF THE PERMEABILITY FACTOR AND ITS INHIBITOR 82

BAUER, D. J.—THE ANTIVIRAL AND SYNERGIC ACTIONS OF ISATIN THIOSEMICARBAZONE AND CERTAIN PHENOXYPYRIMIDINES IN VACCINIA INFECTION IN MICE 105

SURVEY OF PAPERS i

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING.